PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/82, 15/55, 15/53
A01H 1/02

A1

(11) International Publication Number: WO 92/01799

(43) International Publication Date: 6 February 1992 (06.02.92)

(21) International Application Number: PCT/CA91/00255

(22) International Filing Date: 22 July 1991 (22.07.91)

(30) Priority data:
556,917 20 July 1990 (20.07.90) US

(71) Applicant *(for all designated States except US)*: PALADIN HYBRIDS INC. [CA/CA]; 2475 Don Reid Drive, Ottawa, Ontario K1H 8P5 (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : FABIJANSKI, Steven, F. [CA/CA]; 1072 Bladsdell Avenue, Ottawa, Ontario K1K 0C4 (CA). ARNISON, Paul, G. [CA/CA]; 1612 Marcoux Drive, Orleans, Ontario K1E 2K6 (CA).

(74) Agent: HUGHES, ETIGSON, 175 Commerce Valley Drive West, Suite 200, Thornhill, Ontario L3T 7P6 (CA).

(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN + (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: BINARY CRYPTOCYTOTOXIC METHOD OF HYBRID SEED PRODUCTION

(57) Abstract

The invention relates to a method for the preparation of a seed of a plant comprising crossing a male sterile plant and a second plant which is male fertile, and obtaining seed of said male sterile plant, said male sterile plant and said second plant being selected such that said seed has integrated into its genome a first recombinant DNA molecule having a first DNA sequence which encodes a first gene product and a first promoter which is capable of regulating the expression of said first DNA sequence, and a second recombinant DNA molecule which contains a second DNA sequence which encodes a second gene product and a second promoter which is capable of regulating the expression of said second DNA sequence, one of said first and said second recombinant DNA molecules originating from said male sterile plant and the other of said first and second recombinant molecules originating from said second plant, and said first and second gene products cooperating to selectively interfere with the function and/or development of cells of a plant that are critical to pollen formation and/or function of a plant grown from said seed whereby said plant grown from said seed is substantially male sterile. The invention also relates to a method of producing F1 hybrid seed from a plant regenerated from such seed, a method of producing F2 hybrid seed using plants regenerated from the F1 hybrid seed, the products produced from these methods and their use.

+ See back of page

+ DESIGNATIONS OF "SU"

It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

Title: BINARY CRYPTOCYTOTOXIC METHOD OF HYBRID SEED PRODUCTION

FIELD OF THE INVENTION

The invention relates to a process for the preparation of a seed of a plant and to the products produced by the process. The invention also relates to a method of producing F1 hybrid seed from a plant grown from such seed, a method of producing F2 hybrid seed using plants grown from the F1 hybrid seed, the products produced from these methods and their use.

BACKGROUND ART

Production of hybrid seed for commercial sale is a large industry. Hybrid plants grown from hybrid seed benefit from the heterotic effects of crossing two genetically distinct breeding lines with the result that the agronomic performance of the offspring is superior to both parents, typically in vigour, yield, and uniformity. The better performance of hybrid seed varieties compared to open-pollinated varieties makes the hybrid seed more attractive for farmers to plant and thereby commands a premium price in the market place.

Genic male sterility has been utilized in hybrid seed production. Various methods of genic male sterility production and hybrid seed production using male sterile plants are described by the present inventors in published Australian Patent Application Serial No. 611258 and in published PCT Application No. PCT/CA90/00037 by Paladin Hybrids.

Other male sterility systems are disclosed for example in PCT Application No. PCT/WO89/10396 by Plant Genetic Systems.

SUMMARY OF THE INVENTION

The present invention relates to a method for the preparation of a seed of a plant comprising crossing a male sterile plant and a second plant which is male

fertile, and obtaining seed of said male sterile plant, said male sterile plant and said second plant being selected such that said seed has integrated into its genome a first recombinant DNA molecule comprising a first DNA sequence which encodes a first gene product and a first promoter which is capable of regulating the expression of said first DNA sequence, and a second recombinant DNA molecule comprising a second DNA sequence which encodes a second gene product and a second promoter which is capable of regulating the expression of said second DNA sequence, one of said first and said second recombinant DNA molecules originating from said male sterile plant and the other of said first and second recombinant molecules originating from said second plant and said first and second gene products cooperating to selectively interfere with the function and/or development of cells of a plant that are critical to pollen formation and/or function of a plant grown from said seed whereby said plant grown from said seed is substantially male sterile.

Preferably, the male sterile plant is obtained by exposing a plant carrying a male sterile trait to a sterility actuating agent.

Preferably, the first recombinant DNA molecule and second recombinant DNA molecule are located on opposite chromatids of the same chromosome pair and most preferably on opposite chromatids of the same chromosome pair at the same genetic locus such that segregation of the first and second recombinant DNA molecules occurs during meiosis and the chance of recombination of the first and second recombinant DNA molecules to the same chromatid during meiotic crossing over is substantially reduced.

Preferably, the first DNA sequence encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and said second DNA sequence encodes a second gene product which is

said non-toxic substance or encodes a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance.

In accordance with one embodiment of the invention, the male sterile plant has integrated into its genome a first recombinant DNA molecule comprising a first DNA sequence which encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a first promoter which is capable of regulating the expression of said first DNA sequence, the male sterile plant being produced by exposing a plant having said first recombinant DNA molecule integrated into its genome to said non-toxic substance, and wherein the second plant has integrated into its genome a second recombinant DNA molecule comprising a second DNA sequence which encodes a second gene product which is capable of converting a substance endogenous to a plant cell to the non-toxic substance and a second promoter which is capable of regulating the expression of said second DNA sequence. Preferably, the first DNA sequence encodes indole acetamide hydrolase (IamH), the second DNA sequence encodes indole acetamide synthase (IamS) and the first and second promoters are pollen specific promoters.

In accordance with another embodiment of the invention, the male sterile plant has integrated into its genome a first DNA sequence which encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a first promoter which is capable of regulating the expression of said first DNA sequence,and a second DNA sequence which encodes a second gene product which is said non-toxic substance or encodes a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance and a second promoter which is capable of regulating the expression of said second DNA sequence, one of said first promoter and said second promoter being

an inducible promoter which is capable of being activated by an inducer throughout pollen formation, and the other of said first promoter or said second promoter is a pollen specific promoter, the male sterile plant being produced by exposing a plant having said first DNA sequence and said first promoter and said second DNA sequence and said second promoter integrated into its genome to said inducer, and wherein the second plant has integrated into its genome a pollen specific promoter or a constitutive promoter and either of said first or said second DNA sequences which is regulated by the inducible promoter which is integrated into the genome of said male sterile plant.

In particular, the male sterile plant may have integrated into its genome a first DNA sequence which encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function regulated by an inducible promoter which is capable of being activated by an inducer throughout pollen formation, and a second DNA sequence which encodes a second gene product which is said non-toxic substance or a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance regulated by a pollen specific promoter, the male sterile plant being produced by exposing a plant having said first DNA sequence regulated by said inducible promoter and said second DNA sequence regulated by said pollen specific promoter integrated into its genome to said inducer, and wherein the second plant has integrated into its genome said first DNA sequence and a pollen specific promoter or a constitutive promoter.

Preferably, the first DNA sequence encodes IamH, the second DNA sequence encodes IamS. The first DNA sequence may also encode an enzyme which is capable of rendering a protoxin cytotoxic to cells of a plant that are critical to pollen formation and/or function, and the second DNA sequence may encode a protoxin.

The male sterile plant may have integrated into its genome a first DNA sequence which encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function regulated by a pollen specific promoter, and a second DNA sequence which encodes a second gene product which is said non-toxic substance or a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance regulated by an inducible promoter which is capable of being activated by an inducer throughout pollen formation, the male sterile plant being produced by exposing a plant having said first DNA sequence regulated by said pollen specific promoter and said second DNA sequence regulated by said inducer integrated into its genome to said inducer, and wherein the second plant has integrated into its genome said second DNA sequence and a pollen specific promoter or a constitutive promoter. Preferably, the first DNA sequence encodes IamH, and the second DNA sequence encodes IamS. The first DNA sequence may also encode an enzyme which is capable of rendering a protoxin cytotoxic to cells of a plant that are critical to pollen formation and/or function, and the second DNA sequence may encode a protoxin.

It will be appreciated that the male sterile plants containing the first and second DNA sequences may have these DNA sequences located on the same recombinant DNA molecule or on different recombinant DNA molecules.

The invention also relates to a method for producing hybrid seed which comprises cross-pollinating a progeny male sterile plant grown from the seed obtained in accordance with the above described method of the invention, with a suitable male fertile plant which does not contain a first recombinant DNA molecule or second recombinant DNA molecule as hereinbefore described, and harvesting hybrid seed from the progeny male sterile plant. For ease of reference, this method of producing hybrid seed is hereinafter referred to as a binary cryptocytotoxic

method of hybrid seed production.

A method is also provided for producing F2 plants by outcrossing F1 plants grown from the hybrid seed obtained from the progeny male sterile plant. The seed obtained from the F2 plants may be processed to obtain products such as edible oil, etc. Accordingly, the invention also relates to a method of using the seed of an F2 plant obtained in accordance with the methods of the present invention.

According to a preferred embodiment of the invention, the invention provides a method of producing seed of a male sterile plant, comprising:

(a) producing a male sterile plant line comprising

(i) introducing into the genome of one or more plant cells of a pollen-producing plant a first recombinant DNA molecule comprising a DNA sequence which encodes a gene product which when produced in a cell of a plant which is critical to pollen formation and/or function is capable of rendering a non-toxic substance cytotoxic to said cell, preferably said non-toxic substance is a chemical agent, most preferably 2-amino-4-methoxy butanoic acid, a non-toxic analog of glucuronic acid, naphthalene acetamide or indole acetamide, preferably said first recombinant DNA molecule comprises a pollen specific promoter and a selection marker gene which encodes a selection gene product which permits the selection of a plant having said first recombinant DNA molecule integrated into its genome;

(ii) selecting a plant cell into which the first recombinant DNA molecule is stably integrated;

(iii) regenerating from the selected plant cell a plant which carries the male sterile trait;

(iv) increasing the number of plants which carry the male sterile trait to produce a plant line having plants carrying the male sterile trait; and (v) exposing said plant line to the non-toxic substance to render plants of said plant line male sterile;

(b) cross pollinating plants of the male sterile plant line obtained in (a) above with plants of a second plant line having a genome which stably incorporates a second recombinant DNA molecule comprising a second DNA sequence encoding a second gene product which is capable of converting a substance which is endogenous to cells of said second plant line, to said non-toxic substance; a second promoter capable of regulating the expression of said second DNA sequence, preferably a pollen-specific promoter; preferably said first and second recombinant DNA molecules are incorporated into homologous chromosome pairs, and wherein plants of said second plant line are not capable of rendering the non-toxic substance cytotoxic to cells of plants of said second line which are essential to pollen formation and/or function; and

(c) harvesting seed of plants of said male sterile line.

The above mentioned methods of the invention may be used to provide hybridization systems with the following advantages:

(a) Hybrid seed production is not labour intensive and can be achieved on a large scale with commercially acceptable costs;

(b) F1 hybrid seed is fully male fertile;

(c) the population of F2 plants produced by outcrossing F1 plants contain 12.5% male sterile plants, thereby discouraging seed saving or holdback for subsequent planting.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates an embodiment of the process of the invention using the IamH and IamS genes.

Figure 2 illustrates the segregation patterns of the IamH and IamS genes in the F1 and F2 populations when the genes are on the same segregation unit.

Figure 3 illustrates the procedure used for the isolation of the T-DNA gene 1 (the IamS: indole acetamide synthase gene) of the Agrobacterium tumefaciens Ti plasmid derivative pPCV 311 and the construction of a promoterless

version of this gene.

Figure 4 illustrates the procedure used for the isolation of the T-DNA gene 2 (the IamH: indole acetamide hydrolase gene) of the Agrobacterium tumefaciens Ti plasmid derivative pPCV 311 and the construction of a promoterless version of this gene.

Figure 5 illustrates an embodiment of the invention using an IamH gene and an IamS gene.

Figure 6 illustrates the segregation patterns of an IamH gene and an IamS gene in the F1 and F2 populations produced as illustrated in Figure 5.

Figure 7 (7A, 7B, 7C, 7D) are schematic representations describing the production of vectors containing the promoter and promoter regions from clone L4.

Figure 7E shows a schematic representation of the promoter constructs produced as shown, schematically in Figures 7A to 7D.

Figure 8 is a schematic representation describing the production of vectors containing the promoter regions of clone L10.

Figure 9 is a schematic representation describing the production of vectors containing the promoter regions of clone L19.

Figure 10 is a schematic diagram showing the preparation of pPAL0101 and pPALHP101.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned the present invention relates to a method for the preparation of a seed of a plant comprising crossing a male sterile plant and a second plant which is male fertile, and obtaining seed of said male sterile plant, said male sterile plant and said second plant being selected such that said seed has integrated into its genome a first recombinant DNA molecule comprising a first DNA sequence which encodes a first gene product and a first promoter which is capable of regulating the expression of said first DNA sequence, and a second recombinant DNA molecule comprising a second DNA sequence

which encodes a second gene product and a second promoter which is capable of regulating the expression of said second DNA sequence, one of said first and said second recombinant DNA molecules originating from said male sterile plant and the other of said first and second recombinant molecules originating from said second plant and said first and second gene products cooperating to selectively interfere with the function and/or development of cells of a plant that are critical to pollen formation and/or function of a plant grown from said seed whereby said plant grown from said seed is substantially male sterile.

The invention also relates to a method for producing hybrid seed which comprises cross-pollinating a progeny male sterile plant grown from the seed obtained in accordance with the above described method of the invention, with a suitable male fertile plant which does not contain a first recombinant DNA molecule or second recombinant DNA molecule as hereinbefore described, and harvesting hybrid seed from the progeny male sterile plant.

The methods of the invention described herein may be applicable to any species of pollen-bearing plant, particularly species of plants of the genus Brassica and the family Cruciferae (also known as Brassicaceae), the family Solanacae and more particularly other cultivars of Brassica napus. The methods of the invention will be illustrated below with reference to particular embodiments.

AS hereinbefore mentioned the first DNA sequence may encode a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and the second DNA sequence may encode a second gene product which is the non-toxic substance or encode a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance.

A cell and/or tissue of a plant which is critical to pollen formation and/or function includes cells and/or tissues that are instrumental in the development or

function of pollen, including cells and/or tissues from which pollen develops (e.g. premeiotic and uninucleate microspore cells), cells and/or tissues which form part of the male structure in which pollen develops (e.g. anther, tapetum or filament) and pollen itself.

The first DNA sequence may be any identifiable DNA sequences encoding gene products which are capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function. Examples of such a DNA sequence includes a DNA sequence which encodes indole acetamide hydrolase (IamH) which converts naphthalene acetamide to the plant growth regulator alpha naphthalene acetic acid (NAA) which is toxic to developing pollen grains, or converts indole acetamide to indole acetic acid (IAA) which is a plant growth regulator. One source of the enzyme IamH is the bacterium Agrobacterium tumefaciens (Inze, D., et al, 1984, Mol. Gen. Genet. 194:265-74 and Koncz, C. and Schell, J., Molecular and General Genetics, 1986, 204:383-396 re pPCV 311 plasmid derivative). Another source of an enzyme that is genetically equivalent to IamH is the gene coding for indole acetamide hydrolase from Pseudomonas savastanoi (Follin et al. (1985) Mol. Gen. Genet. 201: 178-185).

The first DNA sequence may also encode a gene product which is capable of rendering a non-toxic substance which is a protoxin cytotoxic to a cell of a plant that is critical to pollen formation and/or function. A protoxin has been identified which is inactive against plants but upon enzymatic conversion becomes cytotoxic. (Dotson, S.B. and G.M. Kishore, Isolation of a Dominant Lethal Gene with Potential Uses in Plants In The Genetic Dissection of Plant Cell Processes 1991).

The second DNA sequence may encode a second gene product which is the non-toxic substance or encode a second gene product which converts a substance which is endogenous to a plant cell into the non-toxic substance. For example, a cell may contain a DNA sequence which encodes IamH (which converts indole acetamide to cytotoxic levels of indole

acetic acid), and a DNA sequence which encodes IamS. IamS converts tryptophan which is generally endogenous to plant cells, to indole acetamide which in turn is converted by IamH to cytotoxic levels of indole acetic acid. One source of the enzyme IaMS is the T-DNA gene 1 from the bacterium Agrobacterium tumefaciens (Inze, D., et al, 1984, Mol. Gen. Genet. 194:265-74). Another source of an enzyme that is functionally equivalent to IamS is the gene coding for tryptophan 2-mono-oxygenase from Pseudomonas savastanoi (Follin et al. (1985) Mol. Gen. Genet. 201: 178-185). The second DNA sequence may also encode non-toxic substances such as the above mentioned protoxin.

The promoters used in the methods of the invention may be a pollen specific promoter, an inducible promoter or a constitutive promoter.

A pollen specific promoter is a DNA sequence which regulates the expression of a DNA sequence selectively in the cells/tissues of a plant critical to pollen formation and/or function and/or limits the expression of such a DNA sequence to the period of pollen formation in the plant. ANy identifiable pollen specific promoter may be used in the methods of the present invention.

DNA sequences have been isolated from a plant of the species Brassica napus ssp oleifera cv Westar which are expressed only in microspores and whose expression is essential to microspore function and/or development. A schematic representation of the restriction maps and coding regions of the microspore specific genes identified as L4, L10, L16 and L19 have been detailed in published PCT Application No. PCT/CA90/00037. The complete nucleotide sequence of clones L4, and relevant sequences of L10, L16 and L19 have also been detailed in published PCT Application No. PCT/CA90/00037. The construction of vectors containing pollen specific promoters is illustrated in examples 1 to 6 herein and Figures 7A to 7E, 8 and 9 herein.

Preferably, the pollen specific promoter is a

DNA sequence corresponding to the promoter sequence in the microspore specific genes identified as L4, L10, L16 and L19 above or a functional fragment thereof; or a chimeric promoter sequence containing one or more of a promoter sequence from the microspore specific genes identified as L4, L10, L16 and L19 or portions of such promoter sequences. The L4, L10 and L19 promoter sequences function in tobacco and other plant species. In addition, promoters derived from the L10 gene hybridize to other pollen RNA.

The preferred pollen specific promoters may be used in conjunction with naturally occurring flanking coding or transcribed sequences of the microspore specific genes or with any other coding or transcribed sequence that is critical to pollen formation and/or function.

It may also be desirable to include some intron sequences in the promoter constructs since the inclusion of intron sequences in the coding region may result in enhanced expression and specificity. Thus, it may be advantageous to join the DNA sequences to be expressed to a promoter sequence that contains the first intron and exon sequences of a polypeptide which is unique to cells/tissues of a plant critical to pollen formation and/or function.

Additionally regions of one promoter may be joined to regions from a different promoter in order to obtain the desired promoter activity. Specific examples of chimeric promoter constructs are the chimeric promoters contained in the vectors PAL 1107 (Figure 7a) and PAL 1106 (Figure 7b) as outlined in example 1 and in published PCT Application No. PCT/CA90/00037.

The first promoter or the second promoter used in the method of the invention may be an inducible promoter. An inducible promoter is a promoter that is capable of directly or indirectly activating transcription of a DNA sequence in response to an inducer. In the absence of an inducer the DNA sequence will not be transcribed. Typically the protein factor that binds specifically to an inducible promoter to activate transcription is present in an inactive form which is then directly or indirectly

converted to the active form by the inducer. The inducer may be a chemical agent such as a protein, metabolite (sugar, alcohol etc.),a growth regulator, herbicide, or a phenolic compound or a physiological stress imposed directly by heat, salt, toxic elements etc. or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell such as by spraying, watering, heating,or similar methods. Examples of inducible promoters include the inducible 70 KD heat shock promoter of D.melanogaster (Freeling, M., Bennet, D.C., Maize ADN 1, Ann. Rev. of Genetics 19:297-323) and the alcohol dehydrogenase promoter which is induced by ethanol (Nagao, R.T., et al., Miflin, B.J., Ed. Oxford Surveys of Plant Molecular and Cell Biology, Vol. 3., p. 384-438, Oxford University Press, Oxford 1986). The inducible promoter may be in an induced state throughout pollen formation or at least for a period which corresponds to the transcription of the DNA sequence of the recombinant DNA molecule(s). A promoter that is inducible by a simple chemical is particularly useful since the male sterile plant can easily be maintained by self-pollination when grown in the absence of such a chemical.

It will be appreciated that the term pollen used herein and in particular with reference to the inducible promoter described in the disclosure and claims, includes cells and/or tissues from which pollen develops (e.g. premeiotic and uninucleate microspore cells), cells and/or tissues which form part of the male structure in which pollen develops (e.g. anther, tapetum or filament) and pollen itself.

Another example of an inducible promoter is the chemically inducible gene promoter sequence isolated from a 27kd subunit of the maize glutathione-S-transferase (GST II) gene. Two of the inducers for this promoter are N,N-diallyl-2,2- dichloroacetamide (common name: dichloramid) or benzyl-2-chloro-4-(trifluoromethyl)-5-thiazole-carboxylate (common name: flurazole). In addition a number

of other potential inducers may be used with this promoter as described in published PCT Application No. PCT/GB90/00110 by ICI.

Another example of an inducible promoter is the light inducible chlorophyll a/b binding protein (CAB) promoter, also described in published PCT Application No. PCT/GB90/00110 by ICI.

Inducible promoters have also been described in published Application No. EP89/103888.7 by Ciba-Geigy. In this application, a number of inducible promoters are identified, including the PR protein genes, especially the tobacco PR protein genes, such as PR-1a, PR-1b, PR-1c, Pr-1, PR-Q, PR-S, the cucumber chitinase gene, and the acidic and basic tobacco beta-1,3-glucanase genes. There are numerous potential inducers for these promoters, as described in Application No. EP89/103888.7.

The first or second promoter may be a constitutive promoter. A constitutive promoter is a promoter that functions in all, many, or a variety of cell types including cells/tissues critical to pollen formation and/or function. An example of such a constitutive promoter is CaMV 35S or preferably HP 101 which has been isolated from Brassica napus, which is particularly described below in reference to Figure 10.

The restriction map of a Brassica napus genomic clone (HP 101) deposited January 26, 1990 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD, 20852, USA as pPAL0101/E. coli strain DH5 alpha under accession number ATCC 68210 that contains a constitutively expressed gene is shown in Figure 10 and the fragment of this clone that contains a 5' promoter region along with a portion of transcribed sequence is identified. The fragment was isolated by first cloning the small 2.5 kb Eco RI fragment in pGEM 4Z, and obtaining a subclone that had this fragment inserted in the indicated orientation relative to the polylinker of pGEM 4Z. This subclone, pPAL 0101, was then digested with Eco RI, treated with Klenow fragment, then digested with Bam HI, which releases the

promoter/transcribed region indicated. This fragment was cloned into Hinc II - Bam HI cut pGEM 4Z, resulting in the subclone pPAL HP101. The subclone can be used for the isolation of promoter sequences in vector constructs that utilize a constitutive promoter.

Recombinant DNA molecules containing any of the DNA sequences and promoters described herein may additionally contain selection marker genes which encode a selection gene product which confer on a plant cell resistance to a chemical agent or physiological stress, or confers a distinguishable phenotypic characteristic to the cells such that plant cells transformed with the recombinant DNA molecule may be easily selected using a selective agent. A preferred selection marker gene is neomycin phosphotransferase (NPT II) which confers resistance to kanamycin and the antibiotic G-418. Cells transformed with this selection marker gene may be selected for by testing in vitro phosphorylation of kanamycin using techniques described in the literature or by testing for the presence of the mRNA coding for the NPT II gene by Northern blot analysis in RNA from the tissue of the transformed plant.Transformed plant cells thus selected can be induced to differentiate into plant structures which will eventually yield whole plants. It is to be understood that a selection marker gene may also be native to a plant.

The male sterile plant may be produced by exposing a plant carrying a male sterile trait to a sterility actuating agent. For example, the male sterile plant may be produced by preparing a plant having integrated into its genome a first DNA sequence which encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function regulated by a pollen specific promoter, and exposing the plant to a sterility actuating agent which is the non-toxic substance.

The male sterile plant may also be produced by preparing a plant having integrated into its genome a first DNA sequence which encodes a first gene product which is

capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a first promoter and a second DNA sequence which encodes a second gene product which is the non-toxic substance and a second promoter, the first or second promoter being an inducible promoter which is capable of being activated by a sterility actuating agent i.e.an inducer, throughout pollen formation, and the other of the first and second promoters being a pollen specific promoter, and exposing the plant to the inducer.

The male sterile plant may also be produced by preparing a plant having integrated into its genome a third DNA sequence which is an anti-sense gene which encodes an RNA which substantially interferes with the expression of a sense gene that is critical to pollen formation and/or function or a DNA sequence which encodes a substance which is cytotoxic to cells of a plant that are critical to pollen formation and/or function regulated by an inducible promoter, and exposing the plant to the inducer. The male sterile plant may also be produced by preparing a plant having integrated into its genome a 3rd DNA sequence which is an anti-sense gene which encodes an RNA which substantially interferes with the expression of a sense gene which confers on cells of a plant resistance to a chemical agent or physiological stress regulated by a pollen specific promoter, and exposing the plant to the chemical agent or physiological stress. The 3rd DNA sequence may be located on the same recombinant DNA molecules as the first or second DNA sequences which may be integrated into the genome of the male sterile plant or the 3rd DNA sequence may be located on a different recombinant DNA molecule. These and other methods which may be used for producing male sterility are described in Australian Patent Application Serial No. 611258 and in published PCT Application No. PCT/CA90/00037.

A recombinant DNA molecule containing any of the DNA sequences and promoters described herein may be integrated into the genome of the male sterile plant or

second plant by first introducing a recombinant DNA molecule into a plant cell by any one of a variety of known methods. Preferably the recombinant DNA molecule(s) are inserted into a suitable vector and the vector is used to introduce the recombinant DNA molecule into a plant cell.

The use of Cauliflower Mosaic Virus (CaMV) (Howell, S. H., et al, 1980, Science 208: 1265) and gemini viruses (Goodman, R. M., 1981, J. Gen. Virol. 54: 9) as vectors has been suggested but by far the greatest reported successes have been with Agrobacteria sp. (Horsch, R. B., et al, 1985, Science 227: 1229-1231). Methods for the use of Agrobacterium based transformation systems have now been described for many different species. Generally strains of bacteria are used that harbour modified versions of the naturally occurring Ti plasmid such that DNA is transferred to the host plant without the subsequent formation of tumours. These methods involve the insertion within the borders of the Ti plasmid the DNA to be inserted into the plant genome linked to a selection marker gene to facilitate selection of transformed cells. Bacteria and plant tissues are cultured together to allow transfer of foreign DNA into plant cells then transformed plants are regenerated on selection media. Any number of different organs and tissues can serve as targets for Agrobacterium mediated transformation as described specifically for members of the Brassicaceae. These include thin cell layers (Charest, P. J., et al, 1988, Theor. Appl. Genet. 75: 438-444), hypocotyls (DeBlock, M., et al, 1989, Plant Physiol. 91: 694-701), leaf discs (Feldman, K.A., and Marks, M. D., 1986, Plant Sci. 47: 63-69), stems (Fry J., et al, 1987, Plant Cell Repts. 6: 321-325), cotyledons (Moloney M.M., et al, 1989, Plant Cell Repts 8: 238-242) and embryoids (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75: 30-36). It is understood, however, that it may be desirable in some crops to choose a different tissue or method of transformation.

It may be useful to generate a number of

individual transformed plants with any recombinant construct in order to recover plants free from any position effects. It may also be preferable to select plants that contain more than one copy of the introduced recombinant DNA molecule such that high levels of expression of the recombinant molecule are obtained.

Other methods that have been employed for introducing recombinant molecules into plant cells involve mechanical means such as direct DNA uptake, liposomes, electroporation (Guerche, P. et al, 1987, Plant Science 52: 111-116) and micro-injection (Neuhaus, G., et al, 1987, Theor. Appl. Genet. 75: 30-36). The possibility of using microprojectiles and a gun or other devise to force small metal particles coated with DNA into cells has also received considerable attention (Klein, T.M. et al., 1987, Nature 327: 70-73).

It may also be possible to produce the male sterile plant by preparing a plant carrying a male sterile trait by fusing cells of a plant cell line containing cells having recombinant DNA molecules containing the DNA sequences and promoters described herein with cells of plant species that cannot be transformed by standard methods. A fusion plant cell line is obtained that carries a genetic component from both plant cells. Fused cells that carry the recombinant DNA molecule(s) can be selected and in many cases regenerated into plants that or carry the male sterile trait.

It is contemplated in some of the embodiments of the process of the invention that a plant cell be transformed with a recombinant DNA molecule containing at least two DNA sequences or be transformed with more than one recombinant DNA molecule. The DNA sequences or recombinant DNA molecules in such embodiments may be physically linked, by being in the same vector, or physically separate on different vectors. A cell may be simultaneously transformed with more than one vector provided that each vector has a unique selection marker gene. Alternatively, a cell may be transformed with more

than one vector sequentially allowing an intermediate regeneration step after transformation with the first vector. Further, it may be possible to perform a sexual cross between individual plants or plant lines containing different DNA sequences or recombinant DNA molecules preferably the DNA sequences or the recombinant molecules are linked or located on the same chromosome, and then selecting from the progeny of the cross, plants containing both DNA sequences or recombinant DNA molecules.

Expression of recombinant DNA molecules containing the DNA sequences and promoters described herein in transformed plant cells may be monitored using Northern blot techniques and/or Southern blot techniques. The formation of microspores in plants which contain recombinant DNA molecule(s) such that they are rendered male sterile, is first monitored by visual microscopic examination of the anther structure. As maturation of the flower occurs, anther formation is expected to be delayed or completely inhibited such that no mature pollen grains are formed or released.

Where more than one recombinant DNA molecule of the invention is used to produce a male sterile plant as in the methods of the present invention, the recombinant DNA molecules may be inserted in the same chromosome pair in separate isogenic plant lines. The respective lines are preferably made homozygous for the respective recombinant DNA molecule(s)/gene prior to crossing the lines to produce a male sterile plant. Where a first and a second recombinant molecule are integrated into the same chromosome in the isogenic plant lines, a cross of these lines results in the first and second recombinant DNA molecules being located on separate chromosomes of the same chromosome pair in the male sterile plant. Consequently, when the male sterile plant is crossed with a suitable male fertile plant of a different line, both chromosomes of the chromosome pair segregate into separate F1 progeny with the result that the first and second recombinant DNA molecules are not expressed in the same plant. Thus, the F1 hybrid

seed is fully fertile and thus has restored fertility. If the two recombinant DNA molecules are integrated into different chromosomes in the male sterile plant, then a portion of the F1 hybrid seed will be male sterile since there is a 25% probability of co-segregation of the chromosomes containing both recombinant DNA molecules into the male sterile plant. This latter approach may be advantageous with respect to outcrossing species. When the F1 male fertile plants outcross, a portion of the F2 seed will inherit both chromosomes containing the first and second recombinant DNA molecules and consequently will be male sterile. Where the seed is the commodity of commerce, it is advantageous for seed producing companies to use a scheme for hybrid seed production, where the saving of F2 hybrid seed is discouraged. The outcrossing in the F1 hybrid plants results in partial male sterility in the F2 generation, thereby reducing the seed yield of F2 plants, which is commercially desirable. An example of this method is as follows: a first male sterile plant line incorporating in its genome a recombinant DNA molecule having an IamH gene encoding IamH which converts non-toxic IAM to toxic levels of IAA, may be crossed with a second plant line having a genome incorporating a second recombinant DNA molecule having an IamS gene which converts tryptophan to IAM.

As indicated above, it may be desirable to produce plant lines which are homozygous for a particular gene. In some species this is accomplished rather easily by the use of anther culture or isolated microspore culture. This is especially true for the oil seed crop Brassica napus (Keller and Armstrong, Z. Pflanzenzucht 80: 100-108, 1978). By using these techniques, it is possible to produce a haploid line that carries the inserted gene and then to double the chromosome number either spontaneously or by the use of colchicine. This gives rise to a plant that is homozygous for the inserted gene, which can be easily assayed for if the inserted gene carries with it a suitable selection marker gene for detection of plants

carrying that gene. Alternatively, plants may be self-fertilized, leading to the production of a mixture of seed that consists of, in the simplest case, three types, homozygous (25%), heterozygous (50%) and null (25%) for the inserted gene. Although it is relatively easy to score null plants from those that contain the gene, it is possible in practice to score the homozygous from heterozygous plants by southern blot analysis in which careful attention is paid to the loading of exactly equivalent amounts of DNA from the mixed population, and scoring heterozygotes by the intensity of the signal from a probe specific for the inserted gene. It is advisable to verify the results of the southern blot analysis by allowing each independent transformant to self-fertilize, since additional evidence for homozygosity can be obtained by the simple fact that if the plant was homozygous for the inserted gene, all of the subsequent plants from the selfed seed will contain the gene, while if the plant was heterozygous for the gene, the generation grown from the selfed seed will contain null plants. Therefore, with simple selfing one can easily select homozygous plant lines that can also be confirmed by southern blot analysis.

Two techniques may be used to produce plant lines which carry genes that segregate in a similar fashion or are on the same chromosome or a set of chromosome pairs. One may be a simple crossing strategy in which two transformants that are homozygous for a single inserted gene are crossed to produce F1 seed. The progeny plants from the F1 seed (F1 plant generation) may be crossed with a recipient plant and the segregation of the two inserted genes is determined (F2 plant generation). For example, where the IamH and IamS genes are the inserted genes, the F1 plants grown from the F1 seed will be male sterile. If the original transformants are homozygous for a single inserted gene, when crossed with a non-transformed plant to produce F2 seed, the F2 plants will be 100% male fertile if the two transformants originally used for the production of the F1 seed carried the IamH and the IamS genes on the same

chromosome or in the same linkage group. If the genes are in separate linkage groups or on different chromosomes, a variable degree of male sterility will be seen, in theory 25% of the plants will be male sterile if the genes segregate completely independently of each other. This approach allows for the selection of breeding lines from the homozygous transformed plant lines that contain the IamS and IamH genes which will segregate substantially 100% in the hybrid seed sold for commercial use.

An alternative strategy may make use of extensive genetic maps available for many commercially grown crops and the many easily scoreable markers that are known for most linkage groups or chromosomes. In some cases, linkage groups and chromosomes may be equivalent, whereas in others, there may be more than one linkage group assigned to each chromosome. When there is a marker for each chromosome, identification of the chromosome into which the recombinant gene has been inserted is relatively simple. A cross is made between each individual transformant and a recipient plant that allows for visualization of the marker(s).

If there are scoreable markers that have been localized to each of the chromosomes in the plant, and the markers are scoreable in the generation produced by this cross, one can localize the segregation of the inserted gene with the marker, thereby establishing the chromosomal location of that gene. This therefore allows for the chromosomal or more importantly the linkage group with which the inserted gene segregates. Many crops such as corn, tomato and many cereal crops have extensive genetic maps that allow for the identification of the chromosome containing the inserted gene. It is contemplated that as more detailed chromosome maps are made, especially with the use of RFLP (restriction fragment length polymorphism) maps, the assignment of inserted genes to particular chromosomes will easily be done for most commercial crop species.

As a means of confirmation, or in plant species

where chromosomal markers are not known, it is possible to use a technique called pulse-field electrophoresis (originally described by Schwartz and Cantor, Cell, 37: p67; 1984) to determine if different transformed plants contain inserted genes on the same chromosome. Pulse-field electrophoresis is a technique that can separate large DNA pieces, even chromosomal size, into a reproducible pattern on a gel. When this is done, it is possible to process this gel such that the chromosome spots can be analyzed by southern blotting techniques, localizing the inserted gene to a chromosome spot. When the entire population of primary transformants are analyzed in this fashion, it is a simple task to choose the two transformants that carry the inserted genes on the same chromosome spot.

In one embodiment of the process of the invention for producing hybrid seed a male sterile plant having a genome incorporating a recombinant DNA molecule having a first DNA sequence encoding a protein or polypeptide which renders a non-toxic substance substantially cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a pollen specific promoter is crossed with a second plant which contains a second recombinant DNA molecule having a second DNA sequence which encodes a second gene product which converts a substance which is endogenous to a plant cell to the non-toxic substance. Preferably, the male sterile plant and second plant used in this method are isogenic and each line carries a homozygous loci for the first DNA sequence or the second DNA sequence. Most preferably the first and second DNA sequences are located on the same chromosome pair of the plant lines, such that in any cross of the two lines a single chromosome pair contains both the first and second DNA sequences. The first plant line is made male sterile by exposing the first plant line to the non-toxic substance. The protein or polypeptide encoded by the recombinant DNA molecule incorporated in the genome of the first plant line will render the non-toxic substance toxic in cells of the plant which are critical to pollen

formation and/or function, thus producing a male sterile plant line. The male sterile plant line also preferably has a selection marker gene linked to the first DNA sequence encoding the protein or polypeptide which renders a non-toxic substance cytotoxic to facilitate harvesting of the seeds having cells containing the first and second DNA sequences.

When the first male sterile plant line and the second plant line are crossed, the first male sterile plant line produces seeds having cells containing the DNA sequence encoding a gene product capable of synthesizing the non-toxic substance (e.g. IAM) and the DNA sequence encoding the protein or polypeptide (IamH) which renders the non-toxic substance cytotoxic (e.g. IamH converts IAM to toxic level of IAA). The seed having cells containing the first and second DNA sequences will produce male sterile plants which may be pollinated with a male fertile line to produce commercial hybrid seed. If the first and second DNA sequences are located on the same chromosome or in the same linkage group, the DNA sequences will segregate completely in the F1 hybrid seed and the hybrid seed will be substantially male fertile.

Advantage is taken in the above-mentioned preferred method of the fact that most plant species produce, per plant, many hundreds of seeds. In oilseed Brassica for example, one plant, under normal conditions can produce one thousand seeds. Using the method described above, one can expect a thousand-fold increase in seeds per unit area sprayed with the non-toxic substance. That is to say that, for example, when two isogenic lines are produced that carry the IamS and IamH genes, the first pre-production step involves the use of NAM to cause male sterility in the plant line that carries only the IamH gene. When cross pollinated with the pollen from the plant that contains the IamS gene, one can expect up to one thousand seeds per unit area, each seed capable of growing into a male sterile plant. When these seeds are planted and crossed with a male sterile plant, one can expect one

thousand seeds per unit area. Therefore, if one were to plant one acre of the plant line carrying the IamH gene and the pollinator carrying the IamS gene, this acre would need to be sprayed with NAM. From this one acre however, enough seed would be obtained to grow 1000 acres of male sterile plants and pollinators, and from these 1000 acres, enough hybrid seed with restored fertility would be obtained to plant 1,000,000 acres of hybrid crop. The amount of management required to produce this hybrid seed is reduced over conventional methods because of the pre-production amplification step employed. If the IamH gene is linked to a herbicide resistance gene, one can plant the fields randomly to ensure high rates of cross pollination and use the herbicide to kill the pollinator plants after flowering. This method therefore allows for efficient hybrid seed production over methods where hybrid seed is harvested directly following the first cross pollination.

A preferred embodiment of the above method is described in more detail below with reference to Figures 1 and 2. As illustrated in Figure 1, the method employs two plant lines which are homozygous, respectively, for the IamH gene (plant line A2) and the IamS (plant line A1) genes and otherwise isogenic. These genes are located on the same chromosome pair in each plant, preferably at the same genetic locus or a position such that the chance of a crossing over event is substantially reduced. Accordingly, plants produced from a cross of these two isogenic lines will contain the IamS and the IamH gene respectively on different chromosomes of a single chromosome pair. This will ensure that the two genes will segregate when this plant is crossed with a male fertile plant.

To produce the hybrid seed, a two step procedure is used. The first step involves a pre-production of an isogenic male sterile line, the second step is the hybrid seed production itself.

To accomplish the first step the following approach is used: The two isogenic lines A1 and A2 are planted in rows as shown, and when flowering starts, the

plots are sprayed with NAM (naphthalene acetamide). NAM is also a substrate for the IamH enzyme. This chemical is a non-toxic version of the plant growth regulator NAA, and the action of the IamH gene converts NAM to NAA. Under the control of the pollen specific promoter, the IamH gene only is expressed in pollen of the A2 line, and as such NAA is only made in pollen of the A2 line. Since NAA is a plant growth regulator, normal anther and microspore development is altered, leading to male sterility in the A2 plant line when treated with NAM.

The IamH enzyme can use a number of other substrates, including indole acetamide, indole-3-acetonitrile, conjugates between glucose and IAA and conjugates between myo-inositol and IAA. (Follin et al. (1985) Mol. Gen. Genet. 201: 178-185). Spraying with any of these compounds will produce the same effect as spraying with NAM; namely male sterility in plants expressing the IamH gene.

The plants which contain the IamS gene under the control of the pollen specific promoter (plant line A1) are not affected by the NAM, since they are unable to convert NAM to NAA, therefore these plants remain fully male fertile and can cross pollinate the A2 plants which have now become male sterile after treatment with NAM. On the A2 line, seed is produced that contains both the IamH and IamS genes under the control of pollen specific promoters (plant seed A2/A1).

The seed produced on the A2 line (plant seed A2/A1) is harvested. This harvesting can be done by harvesting specific rows. Alternatively, the IamH gene (A2) may be linked to a gene for herbicide resistance so that the herbicide can be used for the roguing of the plant line A1. Herbicide application takes place after flowering and will kill the A1 plants so that only seed that has the genotype A1/A2 is produced. The seed harvested from such a field will produce substantially 100% male sterile plants. The cross produces plants that express both the IamS and the IamH genes only in the pollen. This leads to

the conversion of tryptophan, an amino acid normally found in plant cells to IAM (indole acetamide) via the IamS activity and finally to IAA (indole acetic acid) via the IamH activity. The molecule IAA is a plant growth regulator that is cytotoxic in greater than normal concentrations in a cell of a plant that is critical to pollen formation and/or function, particularly, in the developing pollen grains or anther of the plant. Since the IAA and the precursor IAM are small molecules that can be transferred from cell to cell via diffusion or active transport, altered growth regulator levels are seen throughout the anther. This altered growth regulator level leads to abnormality in pollen and anther development, producing a male sterile plant. This plant can be pollinated with a male fertile line leading to commercial hybrid seed.

For the production of hybrid seed, the male sterile isogenic line can be planted in rows along side of a suitable male fertile plant, and the hybrid seed produced on the male sterile plant can be harvested. If the IamH gene is linked to a herbicide resistance gene, harvesting of hybrid seed is facilitated by using the herbicide to eliminate pollinator plants after cross pollination. The entire field can then be combined. All seed produced will therefore be hybrid. If the two genes (IamS and IamH) are located on the same chromosome or in the same linkage group, these two genes will segregate completely in the F1 hybrid seed. Since the plants will contain either the IamS or the IamH gene, but not both, the seed produced by this hybrid cross will be substantially 100% male fertile. Therefore the plants grown from the seed of this cross will be fully fertile and set normal levels of seed. The F2 seed that results from the harvest of this field however will contain a variable degree of male sterility, since in theory 12.5% or 2 out of 16 of the plants grown from this F2 seed will contain both the IamS and the IamH genes, as illustrated in Figure 2.

Where there is poor outcrossing, the F2

sterility will be lower than the maximum of 12.5% since a portion of the plants will self and the progeny of selfed plants will not contain both genes. Accordingly, these plants will remain fertile in the F2 generation.

It is contemplated that as a variation of the above particularly preferred method, a number of different ways of producing the toxic molecule specifically in pollen can be envisioned. In all approaches, at least one step in the production of the cytotoxic molecule has to take place specifically within the pollen cells or anthers. For instance, it is possible to use a constitutively expressed IamS gene in a plant and to subsequently cross that plant with a plant that contains the IamH gene under the control of a pollen specific promoter such that IAM is produced in all cells of the plant, but the growth regulator IAA is produced only in pollen cells due to the action of the pollen specific IamH gene. Conversely , it is possible to have IamH constitutively expressed in a plant, and cross this plant with a plant that contains a pollen specific promoter driving the IamS gene. In this situation, the growth regulator IAA is only produced in pollen cells. It should be cautioned that in this case, one cannot use NAM to induce transitory male sterility in the plant that contains the IamH gene, since that application of NAM would be lethal to the plant. In this case then hand pollination would be the preferred way of combining those genes. With regards to these methods the preferred embodiment of the present invention places both the IamH gene and the IamS gene under the control of pollen specific promoters and preferably using the same pollen specific promoter or a pollen specific promoter whose expression substantially overlaps that of the other to each independently drive the expression of these two genes. Additionally, by linking the IamH gene to a selectable agent such as a herbicide, hybrid seed production is greatly facilitated.

Any number of genes could be used to carry out the process and methods of the invention providing that the simultaneous production of two or more enzymatic or

synthetic activities specifically in pollen leads to the production of a substance which is toxic or inhibitory to normal pollen growth or specifically interferes with anther or pollen development. This implies that one or more of these activities could be constitutive in the plant, but that the final combination of all enzyme activities be limited to pollen. It is also envisioned that one of these activities could be inducible by natural or artificial means such that sterility could be induced in plants.

Specifically one embodiment of this method uses a plant line that carries a IamS gene under the control of an inducible promoter and a IamH gene under the control of a pollen specific promoter. These genes are preferably linked, but could be unlinked. When grown under inductive conditions, the plant becomes male sterile and can be pollinated by a suitable male fertile plant. The suitable plant could also carry a IamS gene under the control of a pollen specific promoter such that the progeny of this cross will be male sterile. These plants could then be crossed with a male fertile plant, producing hybrid seed.

The above mentioned embodiment employing a plant that carries a first recombinant DNA molecule having a DNA sequence encoding IamH and an inducible promoter, and a second recombinant DNA molecule having a gene encoding IamS regulated by a pollen specific promoter is described below with reference to Figures 5 and 6. It will be appreciated that the gene encoding IamH and are inducible promoter may be located on the second recombinant DNA molecule having the gene encoding IamS regulated by the pollen specific promoter. As illustrated in Figure 5, the method employs two plant lines which are homozygous, respectively, for the first recombinant DNA molecule and second recombinant DNA molecule (plant line A2) and a first recombinant DNA molecule having a gene encoding IamH and a pollen specific promoter (plant line A1) and are otherwise isogenic. These genes are preferably located at the same genetic locus or a position such that the chance of a crossing over event on corresponding chromatids of a chromosome pair are

substantially reduced. Accordingly, plants produced from a cross of these two isogenic lines will contain the first and second recombinant DNA molecules and the first recombinant DNA molecule on different chromatids of a single chromosome pair. This will ensure that the genes will segregate when this plant is crossed with a male fertile plant.

To produce the hybrid seed, a two step procedure is used. The first step involves a pre-production of an isogenic male sterile line, the second step is the hybrid seed production itself.

To accomplish the first step the following approach is used: The two isogenic lines A1 and A2 are planted in rows as shown, and when flowering starts, the plots are sprayed with an inducer. With reference to Figure 5, the inducer is a chemical inducer. This chemical causes induction of the inducible promoter in the first recombinant DNA molecule such that expression of the gene encoding the IamH occurs in the A2 line. The IamS gene under the control of the pollen specific promoter is expressed only in pollen of the A2 line, and as such IAA is only made in pollen of the A2 line. In the presence of the enzyme IamH, IAM is rendered cytotoxic. Accordingly, normal anther and microspore development is altered, leading to male sterility in the A2 plant line when treated with the chemical inducer.

The plants which contain an IamH gene under the control of the pollen specific promoter (plant line A1) are not affected by the chemical inducer, since these plants do not produce IAM and are thus are unable to produce cytotoxic levels of IAA. Therefore these plants remain fully male fertile and can cross pollinate the A2 plants which have now become male sterile after treatment with the chemical inducer. On the A2 line, seed is produced that contains the first recombinant DNA molecule and the second recombinant DNA molecule from the A2 and the first recombinant DNA molecule from the A1 line (plant seed A2/A1).

The seed produced on the A2 line (plant seed A2/A1) is harvested, more particularly described as discussed above. The seed harvested from such a field will produce substantially 100% male sterile plants which may be pollinated with a male fertile line leading to a commercial hybrid seed as discussed above.

Table 1 outlines a number of possible embodiments according to the process of the invention. It is to be understood that this table does not represent all the possible embodiments but is merely representative of some of the various embodiments. In this Table the IamH and IamS genes are used to illustrate the methods but it is understood that any two DNA sequences which encode gene products which cooperate to selectively interfere with the function and/or development of cells that are critical to pollen formation and/or function may be utilized in the methods of the invention.

The following examples are further provided for illustrative purposes only and are in no way intended to limit the scope of the present invention.

EXAMPLES

Example 1

The construction of 6 vectors containing promoter and promoter fragments from the clone L4 is described in Figure 7 (a,b,c,d,e). The first step in the construction of these vectors was accomplished by first subcloning the Eco R1-Sst 1(nucl.1-2132) fragment containing the first gene of clone L4 (235 base pairs of promoter/exon/intron/second exon) in the commercially available vector pGEM-4Z(Promega Biotech, Madison, WI, USA) using the Eco R1 -Sst 1 sites of the polylinker of this vector. This plasmid was named pPAL 0402. The 2.7Kb Eco RI fragment of clone L4 that contains the third gene (Bp4C) was then cloned into the Eco RI site of pGEM 4Z, leading to a plasmid called pPAL 0411. The plasmid pPAL 0402 was then digested with Eco R1 and the 2.7 Kb Eco R1 fragment from

pPAL 0411(nucl. 5859-8579) that contains the gene number three (Bp4C) from clone L4 was added to it. Clones were recovered that contained this inserted 2.7 Kb Eco R1 fragment in both orientations relative to the promoter region of the first gene. A clone that contained this third gene fragment in a orientation such that the promoter from the third gene was opposite to the promoter in the first gene was chosen and called pPAL 0403. The plasmid pPAL 0403 contains the entire third gene from clone L4 oriented in such a fashion as to have the promoter region immediately adjacent to the 235 basepair promoter region of the first gene in pPAL 0403. This plasmid, pPAL 0403 was digested with Dde I, producing a fragment of approximately 1.9 Kb. The Dde I sites are located at nucleotides 303 and 7366. Because of the orientation of these fragments, digestion with Dde I produces a 1.9 Kb fragment. This 1.9 Kb fragment contains a copy of the third gene (Bp4C) oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 (Bp4A) which is transcribed from left to right, ending in a Dde I site that is located 67 basepairs down stream of the major start site of transcription and precedes that ATG start of translation codon by 2 nucleotides. This 1.9 Kb Dde I fragment was made blunt with Klenow fragment and cloned into the Xba 1 site of the polylinker region of pGEM 4Z previously made blunt ended with Klenow fragment. The resultant plasmid pPAL 0408, was recovered and subsequently was digested with Sal 1 and Sst 1, which releases the cloned Dde 1 fragment bordered by on the left hand side, (nucl 7366) Sal 1 and on the right hand side (nucl 303) of this construct and contains a portion of the polylinker of pGEM 4Z containing the following unique sites: Bam HI, Sma I, Kpn I, and Sst I restriction enzyme sites. This Sal 1 - Sst 1 fragment was cloned into the Sal 1 - Sst 1 sites of PAL 1001. PAL 1001 is the binary vector Bin 19 (described by Bevan, M., Nucleic Acids Res., 1984, 12:8711-8721) to which has been added the nos ter polyadenylation signal as

a 260 bp Sst 1 - Eco R1 fragment isolated from the plasmid pRAJ 221 (available from Clonetech Laboratories, Palo Alto, CA USA) in the Sst 1 - Eco R1 sites of the polylinker region of Bin 19. This nos ter is identified as a stippled box. The binary transformation vector that resulted from the insertion of the Sal I - Sst I fragment of pPAL 0408 into PAL 1001 was named PAL 1107. The details of the construction are shown in Figure 7a. This vector has a copy of the third gene oriented such that the direction of transcription of this third gene is from right to left, fused to the 235 base pair promoter fragment from the first gene of clone L4 which is transcribed from left to right, followed by a polylinker with unique sites for the insertion of DNA which consist of: Bam HI, Sma I, Kpn I and Sst I followed by the nos ter signal. This vector has the feature in that additional 5' non-coding sequences were placed upstream to the 235 base pair core promoter on Bp4A, but these additional 5' sequences were in a opposite orientation. The provision of these sequences in this orientation does not affect the pollen specificity of the core 235 base pair promoter.

In addition to this vector, similarly structured vectors were made which contained essentially the same type of gene promoter arrangement but contained the intron of the first gene (Bp4A) of clone L4. Intron sequences in plant genes have been shown in some cases to play a role in gene expression. This intron containing vector was constructed by making a deletion series of the clone pPAL 0402. pPAL 0402 was first digested with Pst I and Sma I. Exonuclease III was used to unidirectionally digest the DNA as shown (Fig. 8b). After S1 nuclease treatment and repair with Klenow, the plasmid was relegated and clones that have had different portions of the coding regions of gene Bp4A digested out of them were recovered. Deletion subclones were sequenced. One was chosen for vector constructs. This is referred to as deletion 23B. This subclone represented a deletion that has most of the second exon of gene Bp4A removed but contains the intron splice

site and first exon of gene Bp4A. This subclone contains a portion of the clone L4 that extends from nucleotide 1 to nucleotide 1166. To this subclone was added the 2.7 Kb Eco R1 fragment from pPAL 0411 that contains the third gene of L4 (Bp4C) in such an orientation that the direction of transcription of the third gene is from right to left (as in PAL 1107, pPAL 0408), fused to the 235 base pair promoter region from the first gene of clone L4 which is oriented to transcribe from left to right followed by the first exon of gene 1, the entire intron of gene 1 and 33 nucleotides of the second exon of gene Bp4A from clone L4. This plasmid containing deletion 23B and the 2.7 Kb Eco RI fragment containing the third gene fragment was named pPAL 0406. This plasmid was digested with Hind III, which yields a fragment containing a small portion of the promoter of the third gene as well as the entire promoter of the first gene, first exon, intron and a portion of the second exon. This Hind III fragment was inserted into the Hind III site of PAL 1001, resulting in the vector PAL 1106 (deletion 23B derived). This vector has in the following order, A portion of the promoter from the third gene in clone L4, the entire 235 base pair promoter of the first gene in clone L4, followed by the first exon, the intron and a portion of the second exon of gene 1 of clone L4, followed by a polylinker containing the following unique cloning sites: Sal I, Xba I, Bam HI, Sma I, Kpn I and Sst I and the nos ter polyadenylation signal. The construct is shown in Figure 7b.

Example 2

Additional constructs with the promoter regions of the genes contained in clone L4 were done in order to provide a number of suitable vectors that are useful for pollen specific expression of gene sequences. The three genes within clone L4 (Bp4A, Bp4B, Bp4C) show very near-exact DNA homology and this is most apparent between the first (Bp4A) and third (Bp4C) gene. The second gene (Bp4B) is a homologous copy that has undergone sequence changes that have appear to have lead to inactivation. The

extensive similarity between the first, second and third genes in clone L4 is also maintained in the promoter region such that out of the first 235 nucleotides of the first and third gene promoter regions there are only 5 nucleotides that differ between them. Downstream of the TATA box in these two promoters the only difference between them is the presence of one additional nucleotide at the start of transcription. For example, comparison of Promoter 1, Bp4A, partially represented as: .....TATGTTTtAAAA ... with Promoter 3,Bp4C, partially represented as: .....TATGTTTAAAA.... shows that the transcribed region underlined and the single nucleotide difference in lower case. However, within the sequence of the first gene there is a nucleotide change that introduces a Dde I site (nucl 303) in the untranslated 5' leader sequence upstream of the ATG start codon that is not present in the untranscribed leader sequence of the third gene in clone L4. Chimeric promoter constructs were made which utilized this Dde I site in the first gene to combine with sequences from the third gene promoter. The region of the first promoter used for these constructs consisted of the sequences contained between the Sna BI site (nucl 210) near the TATA box to the Dde I site located immediately upstream of the ATG start codon in the first gene (nucleotide 303 is the first nucleotide in the recognition sequence for Dde I). The other region of this chimeric promoter (5' of the TATA box) was a fragment extending from the Eco R1 site of the third promoter (nucleotide 5858) to the Sna B1 site near the TATA box (nucleotide 6272). Therefore to facilitate construction of these pollen specific vectors, the following reconstructions were performed.

The Eco R1 to Dde 1 fragment that encompasses the promoter region of the first gene in clone L4 was isolated by first cutting pPAL 0402 with Dde 1, blunting with Klenow, and then cutting with Eco R1. The 235 base pair fragment corresponding to this region was cloned into the Eco R1 - Sma 1 sites of pGEM 4Z. This plasmid (pPAL 0422), was then cut with Eco R1 and Sna B1. A DNA fragment

that contained the Eco RI to Sna BI portion of the promoter for gene 3 in clone L4 was isolated by digesting pPAL 0411 with Eco R1 and Sna B1. This released an approximately 415 base pair Eco RI (nucl.5858) to Sna BI (nucl. 6272) fragment that represents most of the 5' region of the gene 3 promoter from clone L4 (the Sna B1 recognition site is 2 base pairs downstream of the TATA box). This Eco R1 - Sna B1 fragment was used to replace the shorter Eco R1 - Sna B1 fragment removed for the first promoter subclone (pPAL 0422), reconstructing a promoter fragment of approximately 550 base pairs. This plasmid is referred to as pPAL 0421. This chimeric promoter fragment contains 415 base pairs of the promoter of gene three in clone L 4, followed by approximately 99 Nucleotides of the first gene promoter/untranslated leader sequence.

Example 3

For construction of a pollen specific cassette vector, the following plasmids were first constructed. The first plasmid constructed contained the nos ter polyadenylation signal with a polylinker in front of the nos ter signal. This was accomplished by first isolating from pRAJ 221 the nos ter as a Sst 1 - Eco R1 fragment and this fragment was cloned in pGEM 4Z using the Sst 1 and Eco R1 sites in the polylinker. This subcloned is referred to as pPAL 001. To pPAL 001, a fragment coding for neomycin phosphotransferase (NPT II) derived from the plasmid pRAJ 162 was added to it in the anti-sense orientation as follows: The plasmid pRAJ 162 contains the NPT II gene from the transposon TN 5 inserted as a Sal I fragment and bounded by a polylinker in the plasmid pUC-9 (which was obtained from the Plant Breeding Institute, Cambridge, UK). pRAJ 162 was digested with Hind III and Sma I. The DNA fragment containing the NPT II gene was isolated by elution from an agarose gel. pPAL 001 was digested with Hind III and Sma I and the NPT II gene fragment was inserted. The resultant plasmid was called pPAL 002 and had such orientation of restriction sites and the NPT II gene and nos ter as follows: HIND III, Pst I, Sal I, 3' end NPT II

coding sequence 5'end, Sal I, Bam HI, Sma I, Kpn I, Sst I, nos ter, Eco RI. pPAL 002 was cut with Hind III and the site made blunt ended by the use of Klenow fragment. pPAL 0421 was digested with Hinc II and Pvu II, both of which leave blunt ends, and the promoter fragment was ligated into Hind III cut blunt ended pPAL 002. Plasmids were obtained that contained the promoter in both orientations relative to the nos ter signal. One plasmid was chosen with the proper orientation (5' promoter/anti-sense NPT II/nos ter) and was named pPAL 0419. pPAL 0419 has the following DNA fragments: A small (approx. 130 bp) of pGEM 4Z that contains the SP6 promoter, the 550 base pair chimeric promoter, the NPT II gene in the anti-sense orientation relative to the promoter, followed by the nos ter polyadenylation signal. This entire promoter/NPT II/nos ter construct is excisable by Eco RI. pPAL 0419 was digested with Eco RI, and the promoter NPT II nos ter structure was cloned into BIN 19 using the single Eco RI site in the polylinker of BIN 19. The resultant transformation vector was named PAL 1419. In addition to the anti-sense NPT II gene, the vector contains a constitutive NPT II gene under the control of the nos promoter. This vector therefore confers resistance to kanamycin in all cell types with the exception of pollen cells where the gene expression from the constitutive promoter is inhibited by the anti-sense RNA produced from the promoter/NPT II/nos ter construct contained in PAL 1419.

In order to provide promoter sequences that could be utilized with additional gene constructs, the plasmid pPAL 0419 was digested with Sal I. This digest removes the NPT II coding region and this Sal I digested pPAL 0149 was relegated giving rise to pPAL 0420. pPAL 0420 represents the pollen specific promoter followed by a polylinker for insertion of genes that has the following unique sites: Hinc II, Pst I, Sal I, Bam HI, Sma I, Kpn I, Sst I, followed by the nos ter polyadenylation signal. The entire promoter/polylinker/nos ter construct can be

conveniently excised as a single Eco RI fragment. The details of this construct is shown in Figure 7c.

Example 4

For additional pollen specific promoter constructs, the following approach was used. The intact L4 clone in the lambda cloning vector was digested to completion with the restriction enzymes Sst I and Hha I. The resultant fragments were separated by gel electrophoresis and a 2.65 Kb fragment that contains the promoter/first exon/intron/partial second exon region of gene three in clone L4 and corresponds to nucleotides 4565 to 7210 in the sequence of clone L4 was isolated. This fragment was made blunt ended with Klenow and cloned into the binary transformation vector PAL 1001 previously described. PAL 1001 was first cut with Hind III and made blunt ended with Klenow. Clones containing this fragment (promoter/first exon/intron/partial second exon) were recovered. A clone was chosen that contained this fragment in the proper orientation such that the direction of transcription was towards the nos ter in PAL 1001. This vector was named PAL 1421. This vector contains approximately 1.9kb of upstream promoter region from the gene 3 in clone L4 followed by the first exon, the complete intron and 15 bases of the second exon of gene three followed by a polylinker containing the following unique sites: Sal I, Xba I, Bam HI, Sma I, Kpn I, SstI, and finally the nos ter polyadenylation signal. A variant of this vector was constructed by digesting PAL 1421 with Eco RI and isolating the fragment from this clone that contains the promoter polylinker nos ter sequences but contained less of the upstream region of the promoter. This fragment was re-cloned into PAL 1009. PAL 1009 is a BIN 19 derived vector from which most of the polylinker has been removed. This vector was constructed by digesting BIN 19 with Hind III and Sst I, making these sites blunt ended with Klenow and relegating such that a vector was recovered that contained a single unique Eco RI site for the insertion of fragments. PAL 1009 was digested with Eco RI and the Eco

RI fragment from PAL 1421 that contains a shorter promoter/exon/intron/second exon/polylinker/nos ter structure was added to it. This gave rise to the vector PAL 1422, a vector that is essentially the same as PAL 1421 with the exception that there is less 5' promoter region. It should be noted that both PAL 1421 and PAL 1422 contain the intron from the third gene. For constructs which the presence of the intron may not be desired, intron sequences were removed from PAL 1421 by first digesting PAL 1421 with Eco RI and replacing the promoter/exon/intron/second exon/polylinker/noster structure with the promoter/polylinker/nos ter structure from pPAL 0420 using Eco R1 such that a longer 5' promoter region is reconstructed in the binary transformation vector. The resultant vector was named PAL 1423. The outline of this construction is shown in Figure 7d.

In Figure 7e, a schematic diagram of the relationship of the above described vectors is presented. It should be noted that the vectors outlined in this Figure fall into three categories: 1, vectors which contain 5' upstream promoter regions that are substantially derived from the upstream region of the gene Bp4C (pPAL 0420, PAL 1420, PAL 1423), 2, promoter constructs that contain 5' upstream promoter regions and intron sequences from the gene Bp4C (PAL 1422, PAL 1421) and, 3, promoters which contain a chimeric 5' upstream region in which a portion of the 5' DNA sequence is inverted relative to the arrangement which appears in the genomic clone and uses the promoter fragment of Bp4A as a core promoter structure (PAL 1107, PAL 1106). It should be noted that the functioning of each of these constructs can vary from plant species to plant species and it may be desirable to test a number of these promoter constructs when carrying out certain aspects of this invention.

Example 5

The construction of pollen specific vectors that utilize the promoter regions of clones L10 and L19 was conducted as follows. The construction of the pollen

specific vectors depicted in Figure 8 utilizes promoter regions from clone L10. The start of transcription of clone L10 is located at nucleotide 1. The ATG start codon is located at nucleotides 65-67. The promoter region of this clone was excised by first subcloning the Eco RI - Xba I fragment of the clone that encompasses the entire promoter region and a portion of the first exon (the Xba I site is nucleotide 359 in the DNA sequence). This subclone (pPAL 10EX) was then digested with Hinc II and Nde I. The Nde I site is located immediately upstream of the ATG start codon at nucleotide 62 and the Hinc II site is located at nucleotide number -399. The digestion with these two enzymes releases a DNA fragment of 460 nucleotides which contains 64 nucleotides of untranslated transcribed leader sequence, and 396 nucleotides of 5' promoter region. The Nde I site in this fragment was made blunt ended by the use of Klenow, and this fragment was subcloned into the Hinc II site of the polylinker of pGEM 4Z. Clones were recovered in both orientations and the clone that contained the fragment in the orientation: Hind III, Sph I, Pst I. Hinc II, promoter 64 base pair leader fragment (Nde I blunt/Hinc II, does not cut with either Hinc II or Nde I) Xba I, Bam HI, Sma I, Kpn I, Sst I, Eco RI was chosen and named pPAL 1020. To add additional upstream regions, the Hinc II-HincII fragment that is approximately 1 Kb in length and is immediately upstream of the Hinc II site at position -399 in the DNA sequence was isolated from pPAL 10EX by digestion with Hinc II and gel elution of this fragment. This Hinc II fragment was cloned into the Sma I site of pGEM 4Z. Clones which contained the fragment in both orientations were recovered and a clone that contained the fragment in the following orientation was chosen: Hind III, Sph I, Pst I, Hinc II, Sal I, Xba I, Bam HI, the Hinc II fragment in the same orientation as in the genomic clone, that being right to left, 5'-3' (as a Hinc II/Sma I insertion which does not cut with either enzyme), Kpn I, Sst I, Eco RI. This subclone (pPAL10Hc) was digested with Knp I, made blunt end by the use of Klenow, then digested

with Eco RI. To this cut subclone was added the promoter/untranslated leader sequence of pPAL 1020 by digesting pPAL 1020 with Hinc II and Eco RI, and adding this promoter fragment to the cut pPAL 10Hc. The resultant subclone contained a reconstructed promoter region of clone L10 differing from the intact region by only the filled in Kpn I site used for the joining of the two promoter fragments. This construct was named pPAL 1021. This vector contains in the following order: Hind III, Pst I, Sph I, Hinc II, Sal I, Xba I, Bam HI, the approximately 1 Kb Hinc II fragment joined to the Hinc II-Nde I promoter fragment followed by Xba I, Bam HI, Sma I, Kpn I, Sst I, and Eco RI. This subclone allows for the convenient removal of the promoter region of clone L10 such that the promoter can be easily used in cassette transformation vectors. The outline of this construction is shown in Figure 8. The promoter region of pPAL 1021 was used for the construction of a pollen specific cassette transformation vector by carrying out the following constructs: The plasmid pPAL 1021 was digested with Nco I and Pst I. The plasmid was treated with Klenow and religated. This procedure effectively removed the portion of the polylinker that was 5' to the promoter in pPAL 1021. This plasmid was then digested with Hind III and Sst I, and cloned into the Hind III and Sst I sites of PAL 1001, giving rise to PAL 1121. PAL 1121 has in the following order: the pollen specific promoter of clone L10 (approximately 1.1-1.2 Kb), followed by a polylinker with the following unique sites: Xba I, Bam HI, Sma I, Kpn I, Sst I, followed by the nos ter. The construction of this is outlined in Figure 8.

Example 6

The promoter region of the clone L19 was also used for construction of pollen specific vectors. The construction of these vectors is as shown in Figure 9. Clone L19 has a single pollen specific gene contained with it. The start of transcription in this gene is located at position 1 in the DNA sequence. The ATG start codon is located at nucleotide position 136-138. The only intron is

located at nucleotides 1202-1338, the stop translation codon is located at nucleotides 2025-2027. The end of transcription is located at approximately nucleotide 2074. The entire Eco RI fragment of this clone was subcloned into PGEM 4Z by using the Eco RI site located in the polylinker. The resultant clone was named pPAL 1901. The promoter region of this clone was excised as a single fragment by digesting pPAL 1901 with Bam HI and Eco RV, and a 2177 basepair fragment corresponding to the promoter region was isolated. This fragment covers from nucleotide -2022 (Bam HI) to nucleotide 156 (Eco RV). This promoter fragment contains over 2Kb of 5' upstream region of the promoter in clone L19, 134 basepairs of 5' untranslated leader sequence and 23 basepairs of translated sequence. The Bam HI site in this fragment was made blunt ended by the use of Klenow and cloned into PAL 1001. This step was accomplished by cutting PAL 1001 with Hind III, making this site blunt ended by the use of Klenow and inserting the blunt ended Bam HI - Eco RV fragment in such an orientation that the promoter was oriented 5' to 3' with respect to the polylinker/nos ter polyadenylation signal. This vector was named PAL 1920 and contained within it in the following order: The promoter from clone L19 containing 135 base pairs of 5' untranslated leader sequence, 23 base pairs of translated sequence fused to a polylinker containing a former Hind III site inactivated by blunt ending, Sph I, Pst I, Sal I, Hinc II, Xba I, Bam HI, Sma I, Kpn I, Sst I (the unique cloning sites are underlined), the nos ter polyadenylation signal. This vector is convenient for the insertion of DNA sequences to be transcribed in pollen cells. The outline of this construct is shown in Figure 9.

Example 7

This example describes methods used to transform tobacco and Brassica napus.

For tobacco transformation, the tobacco cultivar, N. tobaccum, cv. Delgold was used. To accomplish this transformation, tobacco leaves less than 8 inches in length were surface sterilized by exposure to ethanol for

5-6 seconds, then subsequent exposure to 1% sodium hypochlorite for a few minutes, usually 5-10 minutes, or until the cut edge of the petiole turned white, then were rinsed several times in sterile distilled water. Leaf segments of approximately 0.5 to 1.0 square centimetres were excised from the sterile leaves, and were cocultured on shoot inducing media for two days with Agrobacterium tumefaciens GV 3101 carrying the Ti plasmid pMP 90 to provide vir functions in trans (described by Koncz, C. and Schell, J., 1986, Mol. Gen. Genet. 204:383-396) carrying the binary vector of interest. The vector is usually a derivative of Bin 19 which contains the NPT II gene driven by the nopaline synthase promoter and terminated by the nos ter for selection of plant cells with kanamycin. Bin 19 is available from Clonetech Laboratories, Palo Alto, CA., U.S.A. Transformed tobacco cells are selected on a shoot-inducing medium containing 0.8% agar, MS salts, B5 vitamins, 3% sucrose, 1 mg per L of benzyladenine, 0.1 mg per L of alpha naphthalene acetic acid, (NAA) 300 µg/ml kanamycin and 500 µg/ml carbenicillin (essentially as described by Horsch et al. 1985, Science, 227:1229-1231). Regenerated shoots are then transferred to a root-inducing medium consisting of B5 medium with 2% sucrose, 500 µg/ml carbenicillin and 0.5 mg/L each of NAA and indoleacetic acid (IAA). Rooted transformants are transferred to a misting chamber containing high humidity, after which the humidity is gradually lowered and plants are subsequently transferred to the greenhouse.

For transformation of Brassica napus, the binary vector containing Agrobacterium strain GV 3101 carrying pMP 90 to provide vir functions in trans is used. Transformation was carried out either using the method described in Moloney, M.M., et al. (Plant Cell Reports (1989) 8:238-242) or, transformation can be carried out with surface sterilized stem epidermal layers. For this procedure, seeds of B. napus L. ssp. oleifera cv. Westar were sown in 'Promix' mixed with 2g/l of the slow-release fertilizer 'Nutricoate' in 8" pots. Plants were grown in

the greenhouse under a 16 photoperiod (using natural and artificial lighting). For coculture and regeneration experiments stem-sections from the top three stem internodes of approximately 1.5 month old plants were used (i.e. those with elongated floral spikes and several open flowers). Intact stem-sections were surface sterilized for 30 seconds in 70% ethanol and 10 minutes in 1% sodium hypochlorite followed by three rinses in sterile distilled water.

For transformation Agrobacterium tumefaciens GV 3101 carrying the Ti plasmid pMP 90 to provide vir functions in trans and the binary vector of choice was grown on YEP media (which consists of 10 gm per L of Yeast Extract, 10 gm per L of Bacto-pepetone and 5 gm per L of NaCl, pH 7.0 containing 100 ugs per mL kanamycin for selection of bacterial cells that contained the binary vectors). Cells were grown from one to two days at 28C. The cells were collected by centrifugation and were resuspended at an approximate density of 10^6 - 10^7 cells per mL in liquid EL which consists of MS micro- and macro-nutrients and B5 vitamins containing 40 mg/L of FeNa-EDTA (obtained from BDH chemicals) and 3% sucrose, 10 mg/L BenzylAdenine, and 0.5 mg/L alpha naphthalene acetic acid (NAA) and 18.8 mM KNO_3 plus 20.6 mM NH_4NO_3. Medium was solidified with 0.8% agar (Sigma) when the EL media was used for solid media plates.

The cell suspension was poured into the bottom of a sterile petri dish and sterilized stems were dissected directly in the bacterial suspensions. The segments were sectioned longitudinally into half segments and cut into approximately 5 mm sections. The dissected segments were placed on filter paper disc on solid EL media for a 3 day coculture under continuous fluorescent light (60 microeinsteins/m^2/sec^2) at 25°C. After a 2-3 day coculture, explants were transferred to solid EL media containing 500 ug/mL carbenicillin, and 100 ug/mL bekanamycin (Sigma). Shoots formed in 4-8 weeks, sections were transferred to fresh solid EL media with carbinicillin and bekanamycin

every 3-4 weeks. Shoots that formed and did not bleach were excised and rooted on PDR media (B5- with 2% sucrose and 0.5 mg/L each of NAA and IAA). In some cases, green non-regenerating callus growing on selective medium was separated from explants and transferred to fresh medium to stimulate regeneration. Transformed plants were placed in misting chamber, and after two - four weeks transferred to the greenhouse. Plants were grown under a 16 hour photoperiod and allowed to flower.

Clonal propagation was used to increase plant lines as well as hand crossing and selection of seedlings from crossed plants on kanamycin containing media. This media consisted of 0.8% agar, one-tenth MS salts and 100 ugs per mL bekanamycin (available from Sigma Chemicals, St. Louis, MO., U.S.A.) with no sucrose in the media. Surface sterilized seeds were used. The seeds were surface sterilized by rinsing in 70% ethanol for a few seconds, soaking in 1% sodium hypochlorate for 15 minutes, followed by rinsing three times in sterile distilled water. Seeds were placed on the surface of the agar in sterile dishes and allowed to sprout. Plants which did not carry the kanamycin gene linked to the antisense gene bleached and died, while those that carried the antisense gene stayed green and were subsequently transferred to soil and allowed to flower.

Example 8

This example describes the isolation of two genes involved in tumour formation in plant tissues following infection with Agrobacterium, the IamS and the IamH genes from the Ti plasmid of the Agrobacterium tumefaciens strain C58. The isolation of the IamH gene is particularly described. The source of DNA coding for these genes was the plasmid pPCV 311. The plasmid pPCV311 is described in: Koncz, C. and Schell, J., Molecular and General Genetics, (1986), 204:383-396, and contains the oncogenic region of the T-DNA plasmid contained in the C58 strain of Agrobacterium. The plasmid pPCV 311, contains a region of T-DNA that when transferred to plant cells causes

tumour formation. This oncogenic region of the T-DNA is entirely contained in the plasmid pPCV-311. This region of DNA contains four genes, that when expressed in plant cells are sufficient for tumour formation. The approximate coding regions of these four genes and the direction of transcription of these four genes are indicated in Figure 4. The other portions of the vector pPCV 311 are not shown in that they are not relative to the following constructions. Additionally, the oncogenic region of the Agrobacterium strain C58 is located on the T-DNA plasmid within that bacterium, commonly referred to as the wild-type nopaline plasmid. A nearly identical oncogenic region is also found in wild type octopine strains which could also be used as a source of genes. The complete nucleotide sequence of an octopine strain oncogenic region is described by Barker et al., Plant Molecular Biology 2:335-350 (1983). The partial sequence obtained from various constructs of genes derived from pPCV 311 was compared to the published nucleotide sequence.

Two genes were isolated from pPCV 311, the IamH and the IamS genes, commonly referred to as genes 2 and 1 respectively. The IamH gene was isolated by first subcloning the indicated Hind III fragment, a fragment that contains all of the coding region of gene 2 and additional 5' sequences that were subsequently removed for the construction of a promoterless version of the gene. The restriction sites mapped in this subclone are shown in Figure 4 and the subclone is referred to as pPAL G2. For the isolation of coding sequences only, pPAL G2 was first split into two smaller clones and the gene later reconstructed. The Xba I - Sma I and Sma I - Sma I fragments shown in Figure 4 were isolated by gel elution and subsequently cloned into the following vectors: The Sma I - Sma I fragment was cloned into pGEM 4Z, giving rise to pPAL 899. The Xba I - Sma I fragment was subcloned into pGEM 7Z, giving rise to pPAL 898. The 5' non-coding sequences of the IamH gene that are present in this subclone were removed in the following fashion: pPAL 898

was digested with Eco RI, the Eco RI site is in the promoter region of the clone, and in this subclone is the only Eco RI site. This digested DNA was then treated with Exonuclease III, and following digestion treated with S1 nuclease and the Klenow fragment of DNA polymerase I. The treated DNA was then cut with Pst I and treated with Klenow fragment in order to make the Pst I site blunt. The linear, digested, blunt ended plasmid was then relegated and used to transform E. coli DH5-alpha according to standard protocols. Subclones were chosen, sequenced and one subclone was chosen that was deleted to 8 nucleotides in front of the ATG start of translation codon. The ATG start codon was determined by comparison of the nucleotide sequence obtained from the deleted subclones to the nucleotide sequence for the octopine strain described by Barker, et al. Plant Molecular Biology 2:335-350 (1983). The nucleotide sequences of both the 5' non-coding and the coding region were nearly identical. This subclone was named pPAL 897, the ATG codon is shown in Figure 4, the direction of transcription in this case would be from right to left in Figure 4. The plasmid contained the 5' half of the coding region from the lamH gene, with the promoter sequences deleted.

The construction of the 3' half of the lamH gene, contained in the plasmid pPAL 898 was carried out as follows. A 3' region of the gene that contains the polyadenylation signal naturally found in the gene was isolated by digestion pPAL 898 with the enzymes Bam HI and Apa I. The digested DNA was treated with Klenow fragment to make it blunt ended and was religated. This gave rise to the subclone pPAL 896, which is a plasmid that contains the 3' half of the IamH gene. To reconstruct the intact lamH gene, pPAL 896 was digested with Hind III and Sma I, and the 3' half gene fragment was isolated by gel elution. pPAL 897 was digested with Sma I and Hind III and the isolated 3' fragment from pPAL 896 was cloned into these sites, reconstructing a promoterless version of the gene that contains the indicated array of restriction sites

flanking the gene. This plasmid was named pPAL 895 and is shown in Figure 4.

Example 9

This example describes the isolation and construction of a promoterless version of the gene 1, IamS: indole acetamide synthase gene of the Ti plasmid of the Agrobacterium tumefaciens strain C58 which procedure is summarized in Figure 3. The gene was isolated from the plasmid pPCV311. The Sma I - Pst I fragment that contains 5' and 3' regions of the lamS gene as well as the coding region was isolated by gel elution and subcloned into a derivative of pGEM 4Z called pGEM-noEco. pGEM-noEco is a plasmid from which the Eco RI site of pGEM 4Z has been removed by cutting with Eco RI and making blunt ended and relegating such that only the Eco RI site was removed. The fragment was inserted in the orientation shown relative to the polylinker. This subclone was called pPAL 889. pPAL 889 was digested with Eco RI, and briefly treated with Exonuclease III, followed by S1 nuclease. The DNA was digested with Sma I and treated with Klenow fragment to make it blunt ended. The DNA was relegated and clones recovered. Some of these clones were chosen, sequenced, and one clone was found which had 5' sequences deleted such that only approximate 15 bases upstream of the ATG start of translation codon remained. This plasmid was named pPAL 888. The Kpn I site at the 5' end of the gene as well as the Pst I site at the 3' end of the gene were both converted to Sal I sites by cutting with Kpn I, end filling with Klenow and adding synthetic Sal I linkers, and repeating the linker addition at the Pst I site such that the entire gene can be excised as a single Sal I fragment. This plasmid was named pPAL 887. This plasmid contains the promoterless version of the lamS gene and contains the array of restriction sites shown that flank the gene as shown in Figure 3.

Example 10

In this example, a pollen specific promoter is used to synthesize the enzyme IamH specifically in pollen

cells. The enzyme has activity that can cause the production of NAA from NAM, the substance NAA functioning as a plant hormone that is substantially toxic to developing pollen grains, while the precursor NAM being relatively non-toxic. For this example, the IamH gene was inserted into the vector PAL 1423. The IamH gene was isolated from pPCV311 as described in Figure 4 and cloned as a Sal I fragment in the Sal I site of PAL 1423, creating PAL 1426. This vector has the IamH gene (T-DNA gene 2) under the control of a pollen specific promoter from clone L4 in the sense orientation. PAL 1426 was used to transform Tobacco as outlined in Example 1.

Example 11

In this example, we use a pollen specific promoter to synthesize the enzyme IamH specifically in pollen cells. The enzyme has activity that can cause the production of NAA from NAM, the substance NAA functioning as a plant hormone that is substantially toxic to developing pollen grains, while the precursor NAM being relatively non-toxic. For this example, the IamH gene was inserted into the vector PAL 1423. The IamH gene was isolated from pPCV311 as described in Figure 4 and cloned as a Bam HI-Sst I fragment in the Bam HI-Sst I sites of PAL 1423, creating PAL 1424. This vector has the IamH gene (T-DNA gene 2) under the control of a pollen specific promoter from clone L4. PAL 1424 was used to transform Tobacco as outlined in example 1.

Example 12

In this example, two isogenic plant lines (A1, A2) were produced that carried either the IamS or the IamH genes. Tobacco plants were transformed with PAL 1426 containing the IamH gene as in Example 4, producing the A2 line. The IamS gene described in Figure 3 was inserted as a Sal I fragment into the vector PAL 1423 in the sense orientation, giving rise to PAL 1425. PAL 1425 was used to transform tobacco as described and tobacco plants were produced that carried PAL 1425. These lines represented the A1 lines. Tobacco plants that contained both PAL 1426

and PAL 1425 were selfed and homozygous A1 and A2 lines were selected.

Example 13

In this example, PAL 1426 (see Example 10) and PAL 1425 were used to transform Brassica napus. Plants lines homozygous for the A1 and A2 genes were selected as in Example 6.

Example 14

In this example, tobacco pollen was harvested from control tobacco plants and from tobacco plants transformed with gene 2 of Agrobacterium tumefaciens, namely the IamH gene, as described in Examples 1 and 10, using PAL 1426. The pollen was then germinated in vitro on matrices containing either NAM or NAA in various concentrations.

In reference to Table 2, pollen from neither the control plants nor the transformed plants germinated in the presence of NAA, which is cytotoxic. The data shown in Table 2 is expressed as the percentage of pollen grains that germinated.

Both control and transformed pollen germinated in the absence of NAA and NAM.

In the presence of NAM, the germination of pollen from control plants was only inhibited at the highest concentration tested (50 ug/ml). By contrast, the germination of pollen from transformed plants was significantly inhibited at all concentrations of NAM tested. Furthermore, pollen tubes that did develop were less than 20% of the length of pollen tubes formed under control conditions. This indicates that the IamH gene is being expressed and that the gene product IamH is functional in transformed plants.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

Table 1

Transient Male Sterility Actuating Agent	Plant Line A2	Plant A1
non toxic substance	psp + IamH	cp + IamS
	psp + IamH	psp + IamS
inducing molecule	psp + IamH and ip + IamS	psp + IamS
	psp + IamH and ip + IamS	cp + IamS
	ip IamH and psp + IamS	psp + IamH
	ip IamH and psp + IamS	cp + IamH
toxic molecule	cp + crg + psp + anti-sense to crg and psp + IamH	psp + IamS
	cp + crg + psp + anti-sense to crg and psp + IamH	cp + IamS
	cp + crg + psp + anti-sense to crg and cp + IamH	psp + IamS

Notes: psp = pollen specific promoter; cp = constitutive promoter; ip = inducible promoter; + = A plus sign between a specified promoter and a specified gene indicates that the promoter regulates the expression of the specified gene; crg = a gene conferring a chemical resistance.

Table 2

Plant	NAA concentration (µg/ml)				NAM concentration (µg/ml)			
	0	12.5	25	50	0	12.5	25	50
501	100	0	1*	0	100	45*	10*	0
503	100	0	0	0	100	70*	36*	0
507	84	0	0	0	84	2.5*	0	0
508	100	0	0	0	100	51	0	0
Control	82	0	0	0	82	65	72	0

*pollen tubes were less than 20% of control length

WE CLAIM:

1. A method for the preparation of a seed of a plant comprising crossing a male sterile plant and a second plant which is male fertile, and obtaining seed of said male sterile plant, said male sterile plant and said second plant being selected such that said seed has integrated into its genome a first recombinant DNA molecule comprising a first DNA sequence which encodes a first gene product and a first promoter which is capable of regulating the expression of said first DNA sequence, and a second recombinant DNA molecule comprising a second DNA sequence which encodes a second gene product and a second promoter which is capable of regulating the expression of said second DNA sequence, one of said first and said second recombinant DNA molecules originating from said male sterile plant and the other of said first and second recombinant molecules originating from said second plant, and said first and second gene products cooperating to selectively interfere with the function and/or development of cells of a plant that are critical to pollen formation and/or function of a plant grown from said seed whereby said plant grown from said seed is substantially male sterile.

2. The method as claimed in claim 1, wherein said first recombinant DNA molecule and said second recombinant DNA molecule are located on opposite chromatids of the same chromosome pair and most preferably on opposite chromatids of the same chromosome pair at the same genetic locus such that segregation of said first and said second recombinant DNA molecules occurs during meiosis and the chance of recombination of the first and second recombinant DNA molecules to the same chromatid during meiotic crossing over is substantially reduced.

3. The method as claimed in claim 1 wherein said first DNA sequence encodes a first gene product which is capable of rendering a non-toxic substance cytotoxic to a

cell of a plant which is critical to pollen formation and/or function and said second DNA sequence encodes a second gene product which is said non-toxic substance or encodes a gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance.

4. The process as claimed in claim 1, wherein the male sterile plant has integrated into its genome said first recombinant DNA molecule comprising a first DNA sequence which encodes a gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a first promoter which is capable of regulating the expression of said first DNA sequence, the male sterile plant being produced by exposing a plant having said first recombinant DNA molecule integrated into its genome to said non-toxic substance, and wherein the second plant has integrated into its genome said second recombinant DNA molecule comprising a second DNA sequence which encodes a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance and said second promoter.

5. The method as claimed in claim 4, wherein the first DNA sequence encodes indole acetamide hydrolase (IamH), the second DNA sequence encodes indole acetamide synthase (IamS) and the first and second promoters are pollen specific promoters.

6. The method as claimed in claim 1, wherein the male sterile plant has integrated into its genome a first DNA sequence which encodes a gene product which is capable of rendering a non-toxic substance cytotoxic to a cell of a plant which is critical to pollen formation and/or function and a first promoter which is capable of regulating the expression of said first DNA sequence and a second DNA sequence which encodes a second gene product

which is said non-toxic substance or encodes a second gene product which is capable of converting a substance endogenous to a plant cell to said non-toxic substance and a second promoter which is capable of regulating the expression of said second DNA sequence, one of the first promoter and the second promoter being an inducible promoter which is capable of being activated by an inducer throughout pollen formation, and the other of said first promoter or said second promoter is a pollen specific promoter, the male sterile plant being produced by exposing a plant having said first DNA sequence and said first promoter and said second DNA sequence and said second promoter integrated into its genome to said inducer, and wherein the second plant has integrated into its genome either of said first DNA sequence or said second DNA sequence which is regulated by said inducible promoter which is integrated into the genome of said male sterile plant and a pollen specific promoter or a constitutive promoter.

7. The method as claimed in claim 6 wherein in said male sterile plant said first promoter is an inducible and said second promoter is a pollen specific promoter, and wherein the second plant has integrated into its genome the first DNA sequence and a pollen specific promoter or a constitutive promoter.

8. The method as claimed in claim 6 wherein in said male sterile plant said first promoter is a pollen specific promoter and said second promoter is an inducible promoter and wherein the second plant has integrated into its genome the second DNA sequence and a pollen specific promoter or a constitutive promoter.

9. The method as claimed in claim 7 or 8 wherein the first DNA sequence encodes IamH and the second DNA sequence encodes IamS.

10. The method as claimed in claim 7 or 8 wherein the first DNA sequence encodes an enzyme which is capable of rendering a protoxin cytotoxic to cells of a plant that are critical to pollen formation and/or function and the second DNA sequence encodes a protoxin.

11. A method for producing hybrid seed which comprises cross-pollinating a progeny male sterile plant grown from the seed obtained using the method as claimed in claim 1, with a suitable male fertile plant which does not contain a first recombinant DNA molecule or second recombinant DNA molecule and, harvesting hybrid seed from the progeny male sterile plant.

12. A seed obtained using the method claimed in claim 1.

13. A method of using the seed obtained using the method claimed in claim 1 comprising crossing a plant grown from said seed with a plant of another line to produce F1 seed.

14. The F1 seed produced by the method as claimed in claim 13.

15. A method of using the seed claimed in claim 14 comprising crossing a plant grown from said F1 seed with a plant to produce F2 seed.

16. The F2 seed produced by the method claimed in claim 15, or any extract thereof.

17. The process as claimed in claim 5 wherein said pollen specific promoters are selected from the group comprising a promoter sequence of a gene from Brassica napus which is essential to pollen formation and/or function and expressed exclusively in one or more of said cells/tissues, and/or any allelic, homologous or other

functional variant or portion of said promoter sequence, more preferably said promoter comprises a promoter sequence of a gene in clone L4, L10, L16 or L19, most preferably the promoter region of gene Bp4A of clone L4 comprising nucleotides 1-307, the promoter region of gene Bp4C of clone L4 comprising nucleotides 4565-6272, the promoter region of clone L10 comprising nucleotides -789-64, the promoter region of clone L19 comprising nucleotides -2022-156, and/or allelic, homologous or other functional variants or portions of said promoter regions which homoduplex therewith.

18. The process as claimed in claim 5 wherein said pollen specific promoters are chosen from the group comprising substantially the sequence of nucleotides 1-235, 6063-6297, 1-303, 6063-6364, 4565-6272, 5859-6272, 5859-6297, 5859-6364, 4565-7210, 5859-7210 or 5859-6163; and portions of said promoter which function as pollen specific promoters or probes for isolating homologous pollen specific promoters; and allelic, homologous or other variants of said promoter fragment which homoduplex therewith and function as pollen specific promoters.

19. The process as claimed in claim 5 wherein said pollen specific promoters are chosen from the group comprising substantially the sequence of nucleotides -399-62 or -789-62; and portions of said promoter which function as pollen specific promoters or probes for isolating homologous pollen specific promoters; and allelic, homologous or other variants of said promoter which homoduplex therewith and function as pollen specific promoters.

20. The process as claimed in claim 5 wherein said pollen specific promoters are chosen from the group comprising the sequence of nucleotides -2022-135 or -2022-156; and portions of said promoter fragment which function as pollen specific promoters or probes for

isolating homologous pollen specific promoters; and allelic, homologous or other variants of said promoter which homoduplex therewith and function as pollen specific promoters.

21. The process as claimed in claims 5 wherein said pollen specific promoters are chosen from the group comprising an upstream region consisting substantially of the inverted sequence of nucleotides 5859 to 7366 of gene Bp4C and a downstream region consisting substantially of the sequence of nucleotides 1 to 303 of gene Bp4A, an upstream region consisting substantially of the inverted sequence of nucleotides 5859 to 6163 of gene Bp4C and a downstream region consisting substantially of the sequence of nucleotides 1 to 1166 of gene Bp4A, an upstream region consisting substantially of the sequence of nucleotides 5859 to 6272 of gene Bp4C and a downstream region consisting substantially of the sequence of nucleotides 210 to 303 of gene Bp4A, or an upstream region consisting substantially of the sequence of nucleotides 4565 to 6272 of gene Bp4C and a downstream region consisting substantially of the sequence of nucleotides 210 to 303 of gene Bp4A; and any portion of said promoter fragment which functions as a pollen specific promoter or as a probe for isolating homologous pollen specific promoters; and any allelic, homologous or other variant of said promoter fragment which homoduplexes therewith and functions as a pollen specific promoter.

22. The process as claimed in claims 5 wherein said pollen specific promoters are chosen from the group comprising nucleotide sequences which are identical or substantially homologous to:

(a) nucleotide sequences selected from the group of conserved nucleotide sequences of clone L10 consisting of nucleotides -524 to -510, nucleotides -189 to -179, nucleotides -456 to -446, nucleotides -446 to -438, nucleotides -14

to -6, nucleotides -6 to 8, nucleotides -52 to -43, and nucleotides -138 to -126;

(b) nucleotide sequences selected from the group of conserved sequences of clone L19 consisting of nucleotides -837 to -823, nucleotides -2013 to -2003, nucleotides -2001 to -1993, nucleotides -456 to -446, nucleotides -1010 to -1001, nucleotides -342 to -329, nucleotides -1809 to -1797, and nucleotides -1863 to -1854;

(c) nucleotide sequences selected from the group of conserved sequences of clone L19 consisting of nucleotides -233 to -210, nucleotides -873 to -847, and nucleotides -1185 to -1153; or

(d) nucleotide sequences selected from the group of conserved nucleotide sequences of clone L4 consisting of nucleotides 38-67 and 6100-6129, nucleotides 168-200 and 6230-6262, and nucleotides 209-233 and 6271-6295.

23. A method of producing seed of a male sterile plant comprising:

(a) producing a male sterile plant line comprising

(i) introducing into the genome of one or more plant cells of a pollen-producing plant a first recombinant DNA molecule comprising a DNA sequence which encodes a gene product which when produced in a cell of a plant which is essential to pollen formation and/or function is capable of rendering a non-toxic substance cytotoxic to said cell, preferably said non-toxic substance is a chemical agent, most preferably 2-amino-4-methoxy butanoic acid, a non-toxic analog of glucuronic acid, naphthalene acetamide or indole acetamide, preferably said first recombinant DNA molecule comprises a pollen specific promoter and a selection marker gene which encodes a selection gene product which permits the selection of a plant having said first

recombinant DNA molecule integrated into its genome;

(ii) selecting a plant cell into which the first recombinant DNA molecule is stably integrated;

(iii) regenerating from the selected plant cell a plant which carries the male sterile trait;

(iv) increasing the number of plants which carry the male sterile trait to produce a plant line having plants carrying the male sterile trait; and

(v) exposing said plant line to the non-toxic substance to render plants of said plant line male sterile;

(b) cross pollinating plants of the male sterile plant line obtained in (a) above with plants of a second plant line having a genome which stably incorporates a second recombinant DNA molecule comprising a second DNA sequence encoding a second gene product which is capable of converting a substance which is endogenous to cells of said second plant line, to said non-toxic substance; a second promoter capable of regulating the expression of said second DNA sequence, preferably a pollen-specific promoter; preferably said first and second recombinant DNA molecules are incorporated into homologous chromosome pairs, and wherein plants of said second plant line are not capable of rendering the non-toxic substance cytotoxic to cells of plants of said second line which are essential to pollen formation and/or function; and

(c) harvesting seed of plants of said male sterile line.

24. A method of producing seed of a plant comprising crossing a female parent plant which is or can be rendered transiently male sterile with a male parent plant, and obtaining seed of said female parent plant, said female

parent plant preferably obtained by exposure to a sterility actuating agent, said female parent plant having a first recombinant DNA molecule comprising a first DNA sequence which encodes a first gene product and a first promoter which is capable of regulating the expression of said first DNA sequence, and said male parent plant having a second recombinant DNA molecule comprising a second DNA sequence which encodes a second gene product and a second promoter which is capable of regulating the expression of said second DNA sequence, said first and second gene products preferably different gene products, said first and second recombinant DNA molecules preferably located on chromosomes of the same chromosome pair, each of said first and second recombinant DNA molecules preferably located on both chromosomes of the same chromosome pair, said first and second promoters selected from the group consisting of a pollen specific promoter and a constitutive promoter, at least one of said first and second promoters preferably being a pollen specific promoter, said first and second gene product cooperating to selectively interfere with the function or development of cells of a plant that are critical to pollen formation or function in a plant grown from said seed such that said plant grown from said seed is substantially male sterile only when both said first and said second DNA sequences are expressed in said cells, preferably one of said first and second gene products is a non-toxic substance or is capable of producing said non-toxic substance from a substance endogenous to a plant cell and the other of said first and second gene products is capable of rendering said non-toxic substance cytotoxic to said cell, more preferably one of said first and second DNA sequences is regulated by a pollen specific promoter and encodes a gene product that is capable of producing said toxic substance form said non-toxic substance upon exposure of said female parent plant to the non-toxic substance such that said female parent plant is transiently male sterile alternatively, other forms of transient male sterility may be used to produce the female parent plant, passively as in

the case of a scheme calling for sterility actuating agents or actively as in the case where the female parent plants used in the crosses to produce the seed of the female plant and the hybrid seed, are transformed with and are homozygous for recombinant DNA molecules which restore the function of the gene(s) encoding the male sterile trait.

FIGURE 1

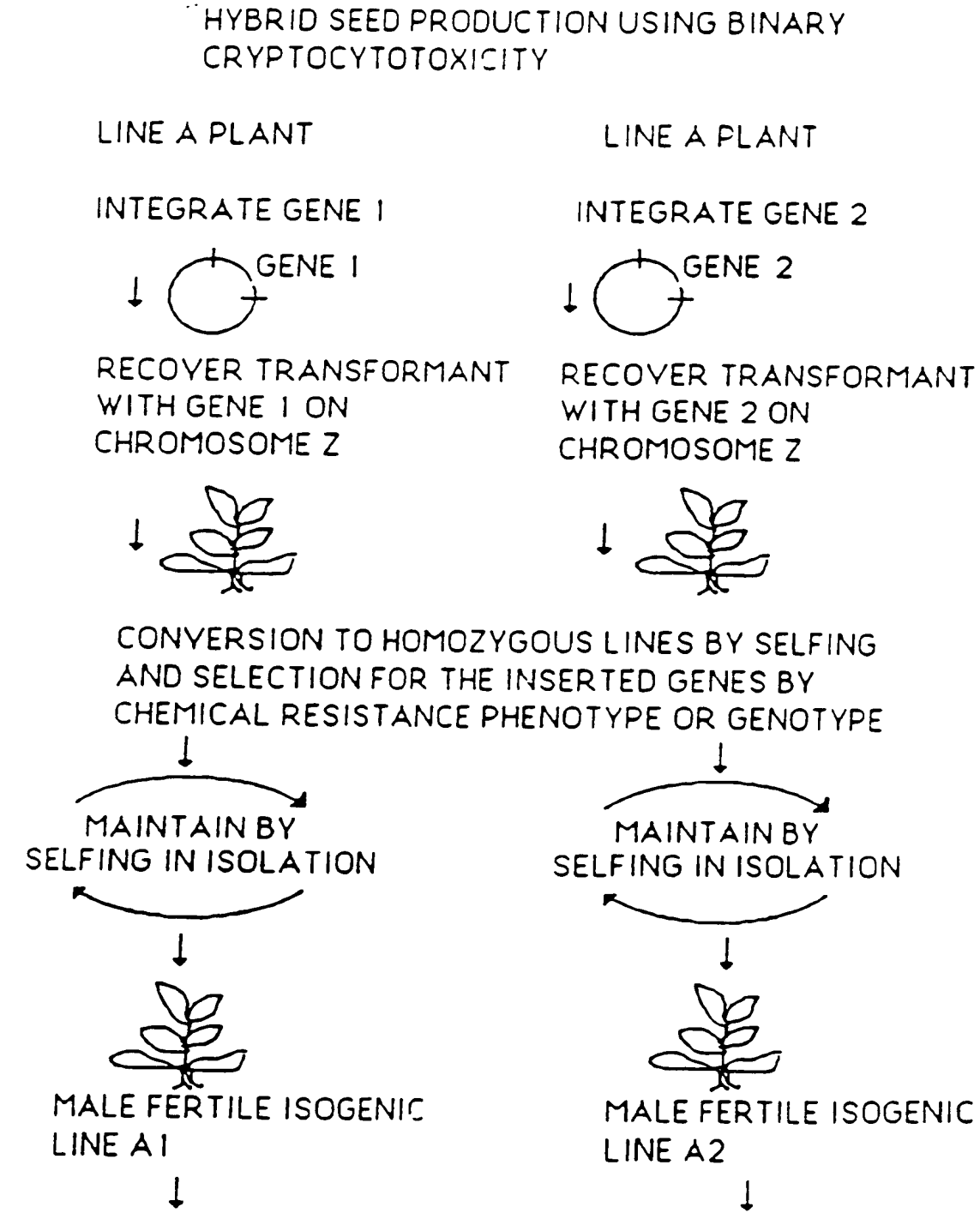

HYBRID SEED PRODUCTION USING BINARY CRYPTOCYTOTOXICITY
LINE A PLANT
INTEGRATE GENE 1
GENE 1
RECOVER TRANSFORMANT WITH GENE 1 ON CHROMOSOME Z
LINE A PLANT
INTEGRATE GENE 2
GENE 2
RECOVER TRANSFORMANT WITH GENE 2 ON CHROMOSOME Z
CONVERSION TO HOMOZYGOUS LINES BY SELFING AND SELECTION FOR THE INSERTED GENES BY CHEMICAL RESISTANCE PHENOTYPE OR GENOTYPE
MAINTAIN BY SELFING IN ISOLATION
MAINTAIN BY SELFING IN ISOLATION
MALE FERTILE ISOGENIC LINE A1
MALE FERTILE ISOGENIC LINE A2

FIGURE 1 (CONT'D)

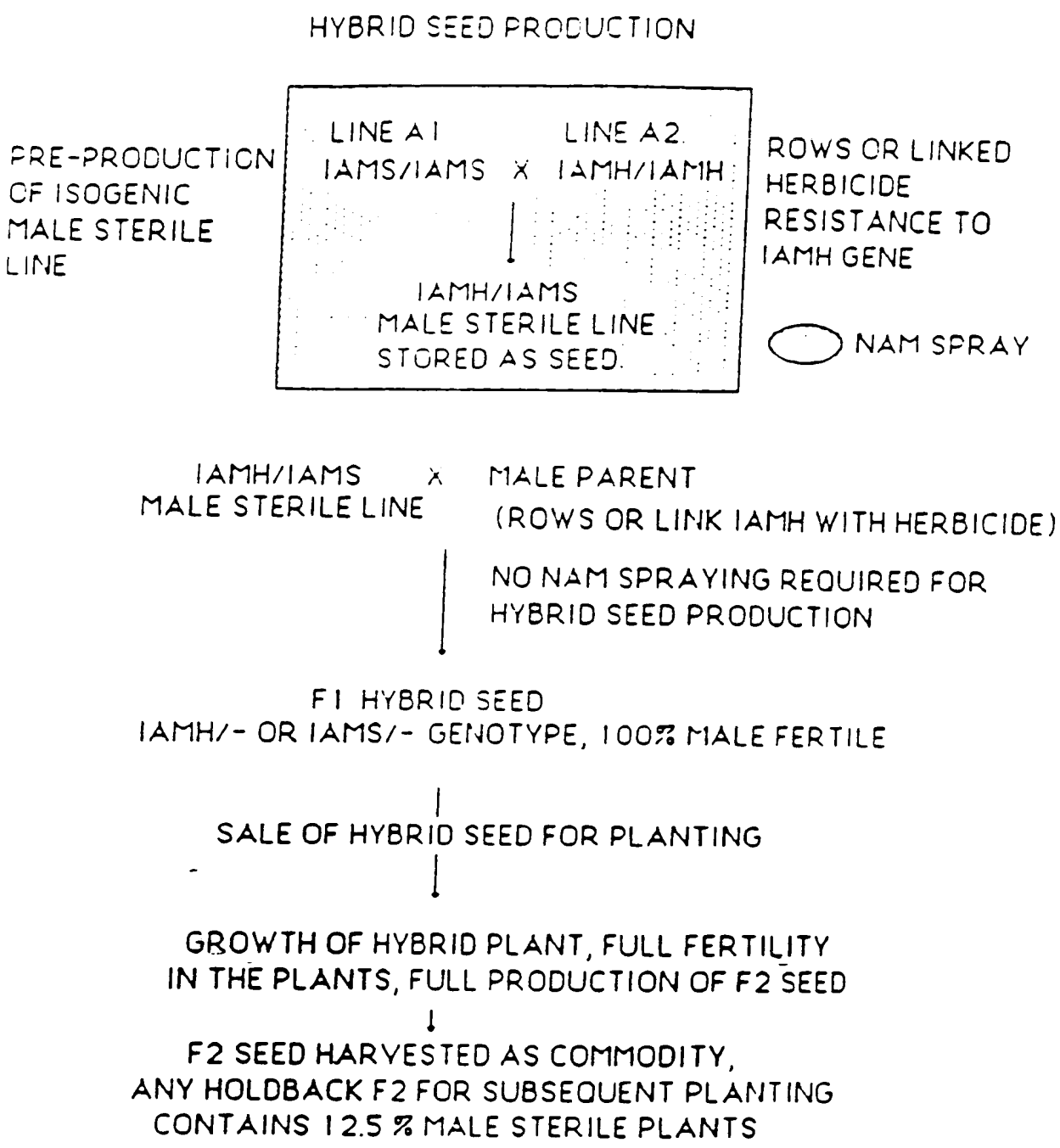


FIGURE 2

SEGREGATION OF BINARY CRYPTOCYTOTOXICITY GENES IF BOTH GENES ARE LOCATED ON THE SAME CHROMOSOME OF A CHROMOSOME PAIR IN THE ISOGENIC MALE STERILE LINE

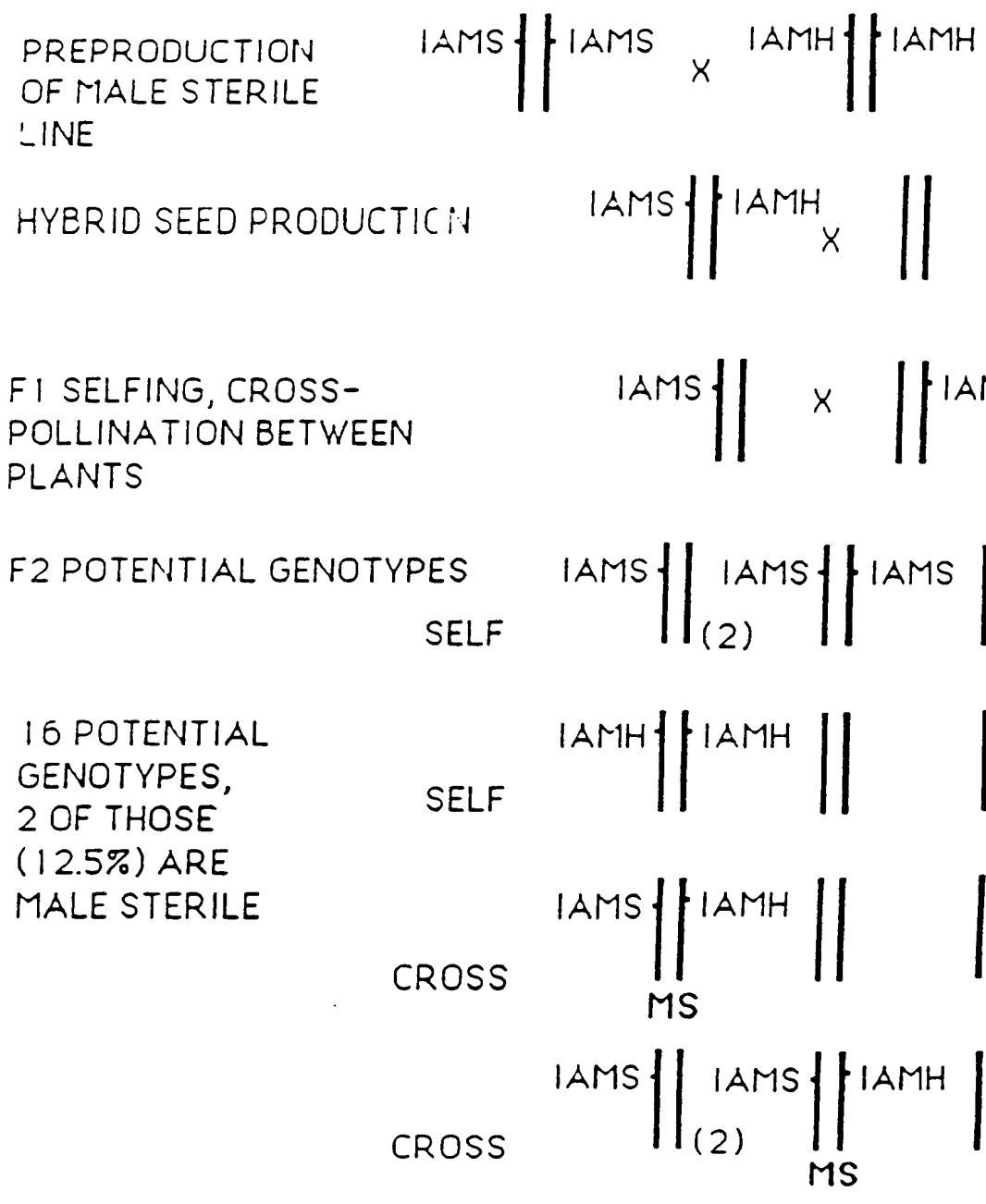
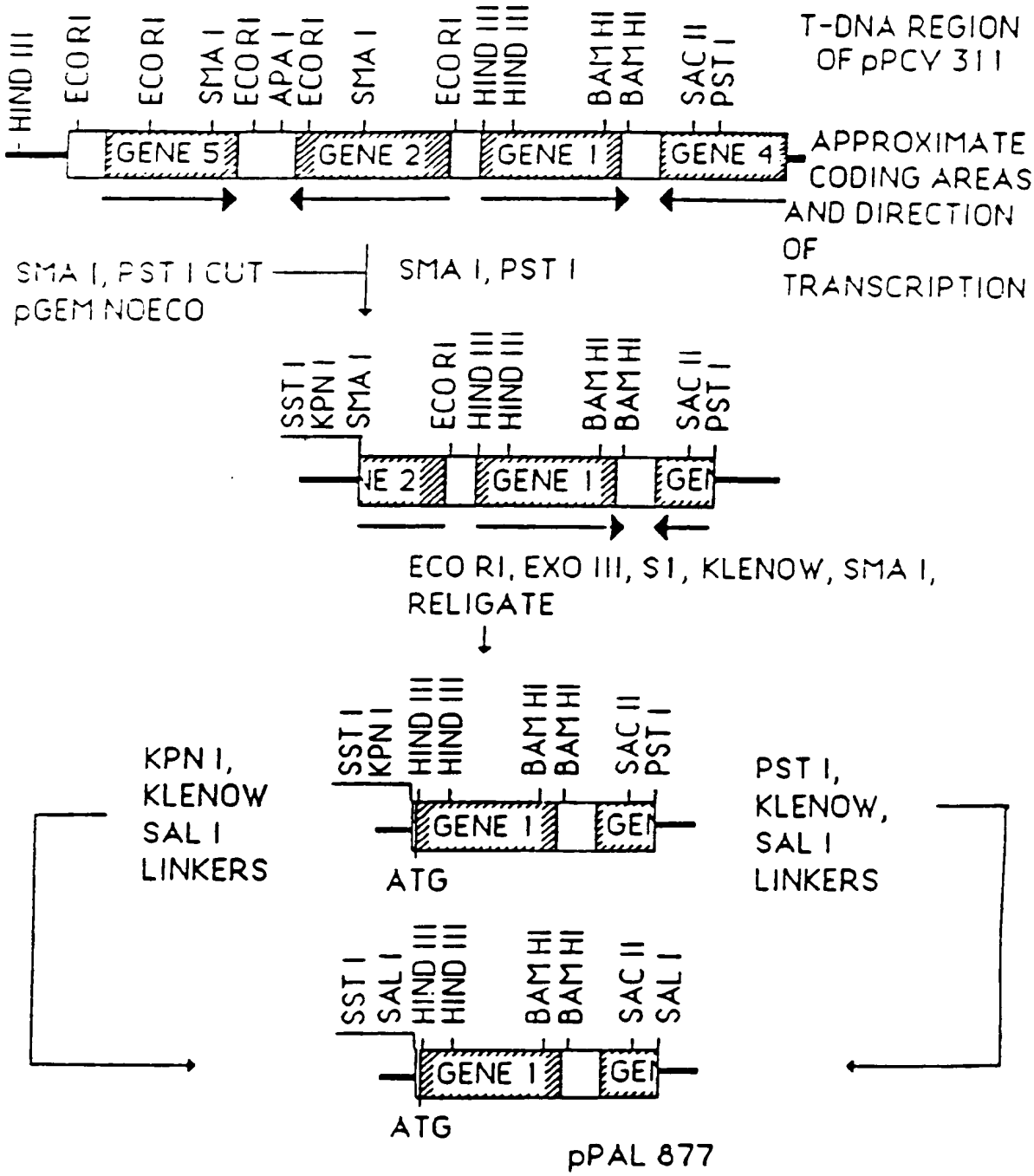


FIGURE 3



SUBSTITUTE SHEET

FIGURE 4

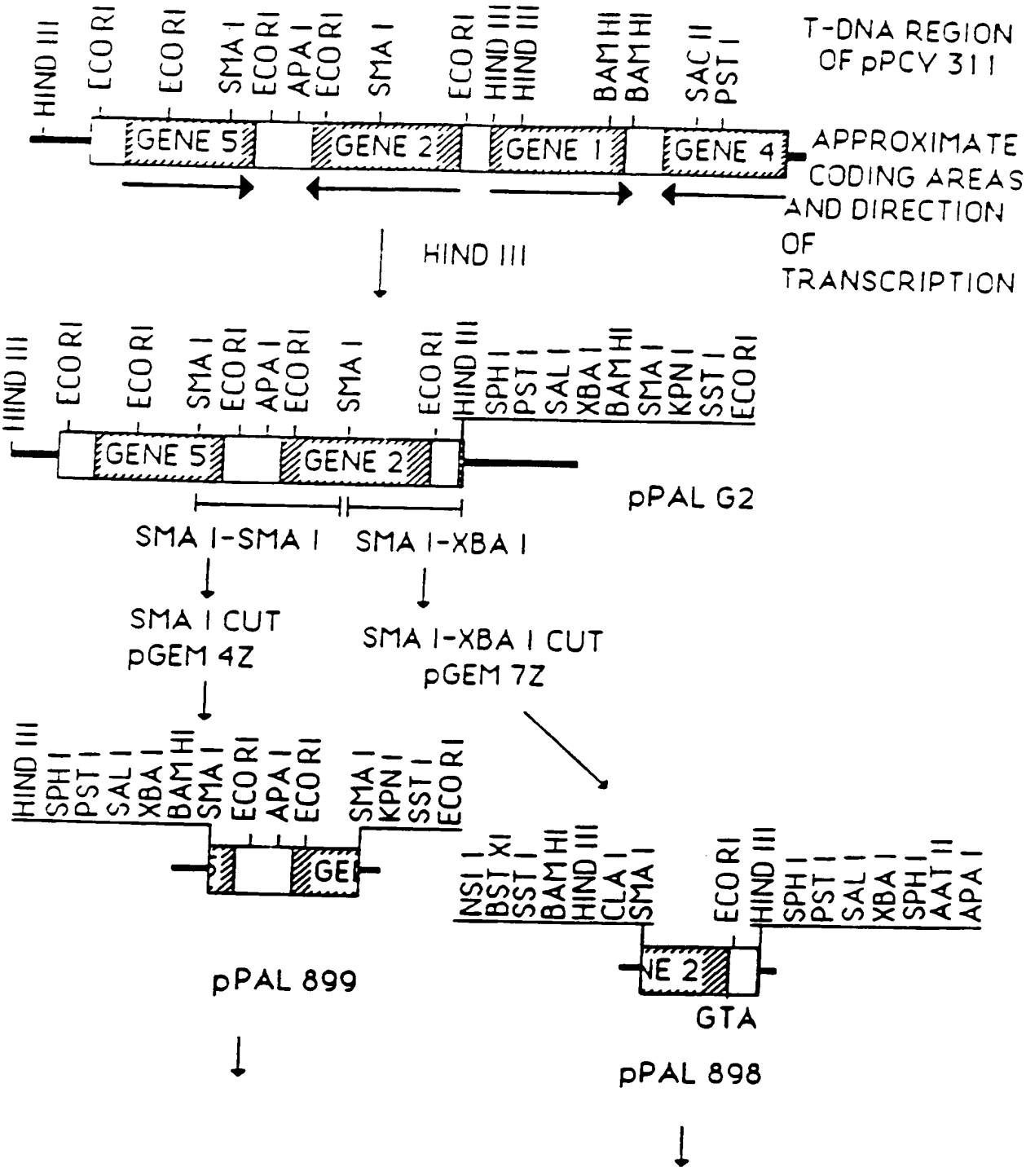

HIND III
ECO RI
ECO RI
SMA I
ECO RI
APA I
ECO RI
SMA I
ECO RI
HIND III
HIND III
BAM HI
BAM HI
SAC II
PST I
T-DNA REGION OF pPCV 311
GENE 5
GENE 2
GENE 1
GENE 4
APPROXIMATE CODING AREAS AND DIRECTION OF TRANSCRIPTION
HIND III
HIND III
ECO RI
ECO RI
SMA I
ECO RI
APA I
ECO RI
SMA I
ECO RI
HIND III
SPH I
PST I
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
ECO RI
GENE 5
GENE 2
pPAL G2
SMA I-SMA I
SMA I-XBA I
SMA I CUT pGEM 4Z
SMA I-XBA I CUT pGEM 7Z
HIND III
SPH I
PST I
SAL I
XBA I
BAM HI
SMA I
ECO RI
APA I
ECO RI
SMA I
KPN I
SST I
ECO RI
GE
pPAL 899
NSI I
BST XI
SST I
BAM HI
HIND III
CLA I
SMA I
ECO RI
HIND III
SPH I
PST I
SAL I
XBA I
SPH I
AAT II
APA I
NE 2
GTA
pPAL 898

FIGURE 4 (CONT'D)

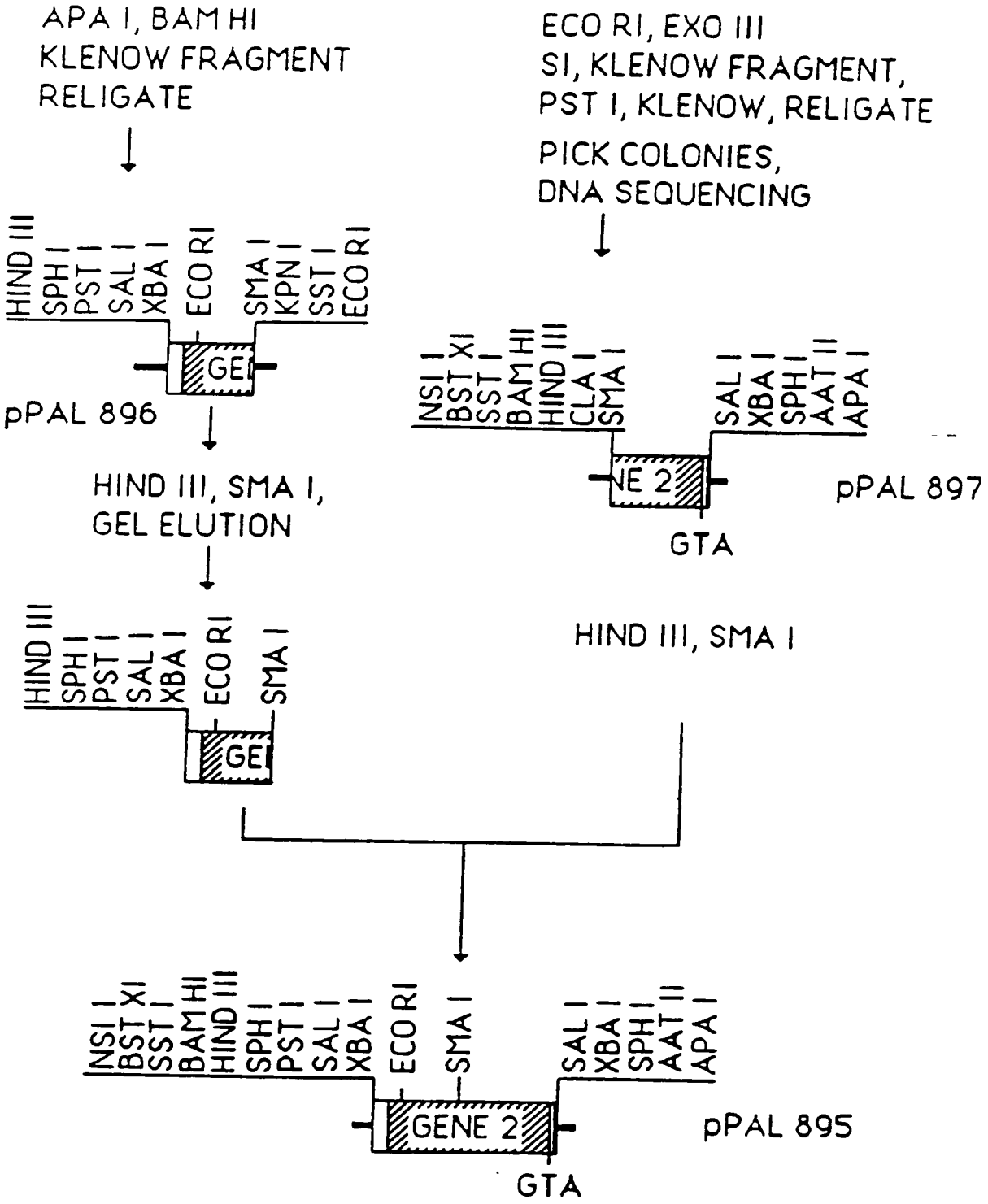


FIGURE 5

HYBRID SEED PRODUCTION USING BINARY CRYPTOCYTOTOXICITY

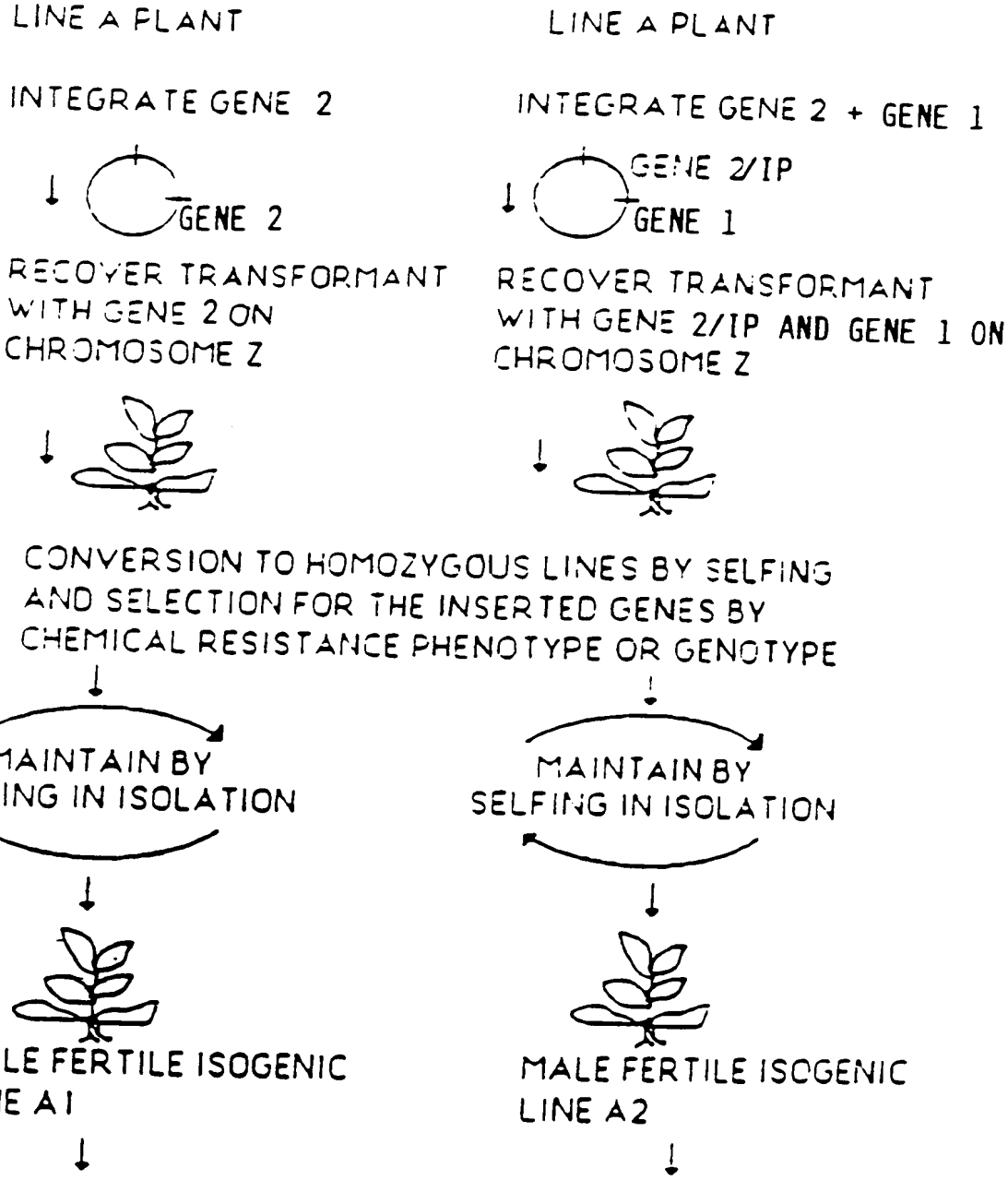
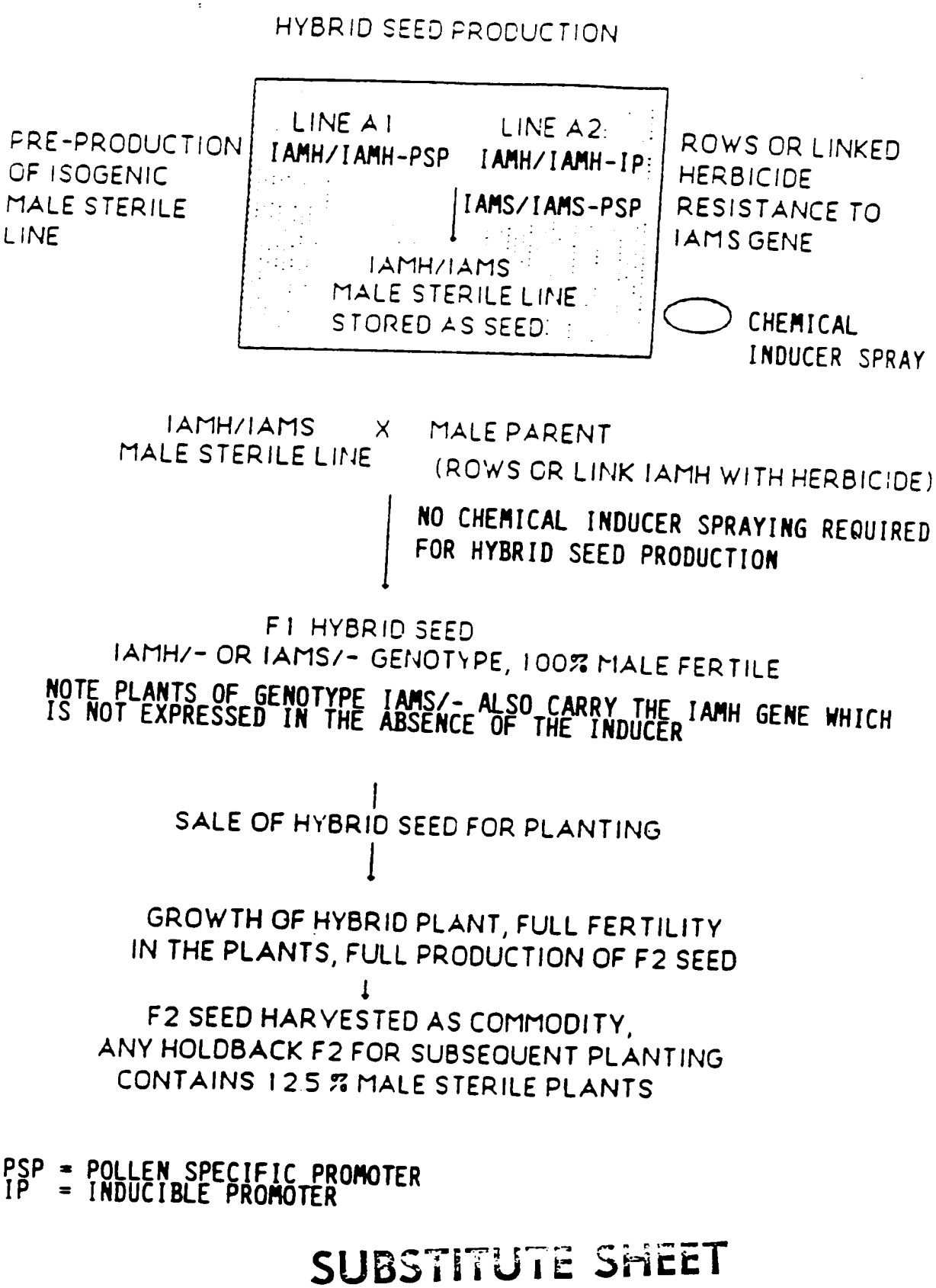


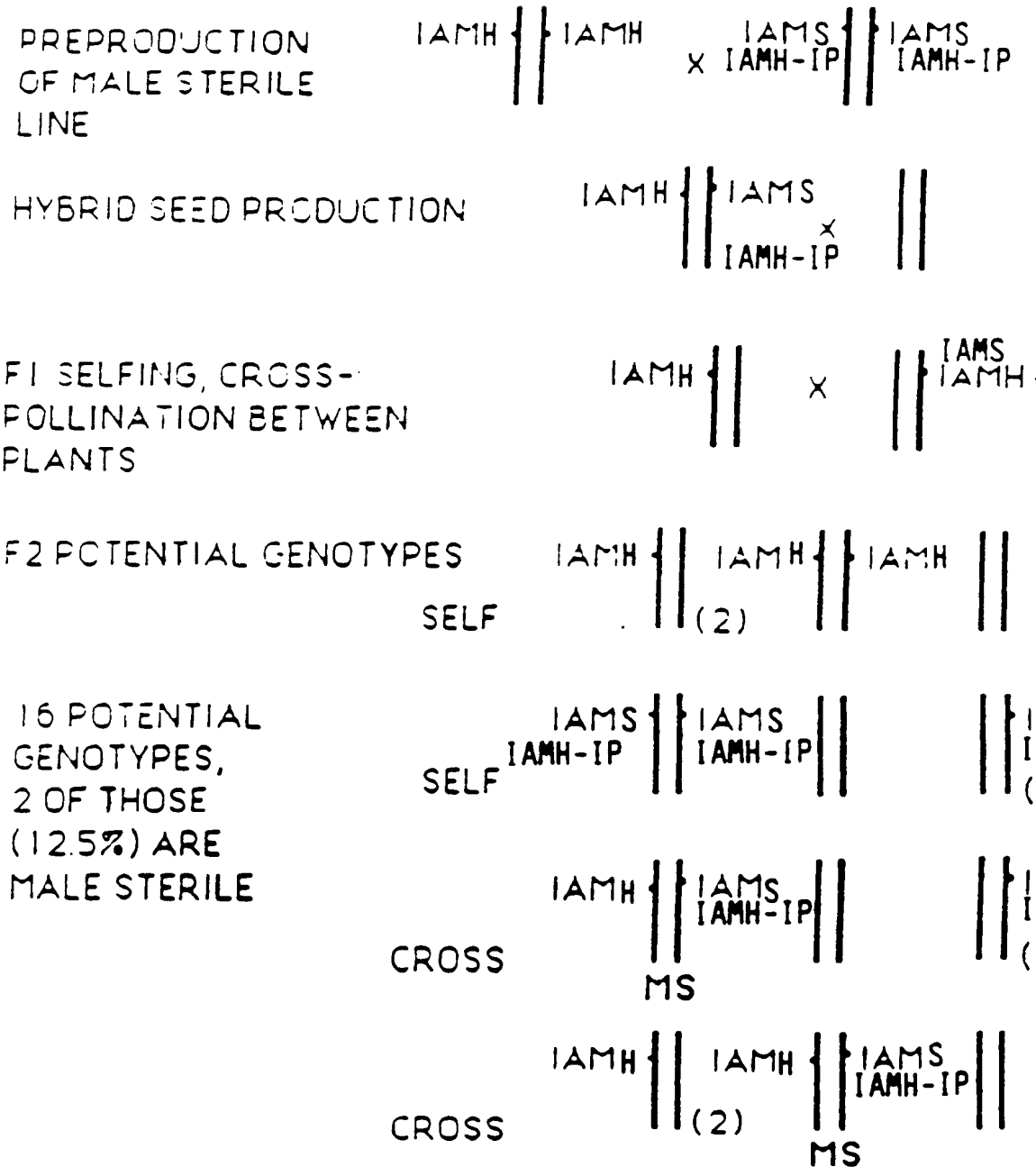
FIGURE 5 (CONT'D)



PSP = POLLEN SPECIFIC PROMOTER
IP = INDUCIBLE PROMOTER

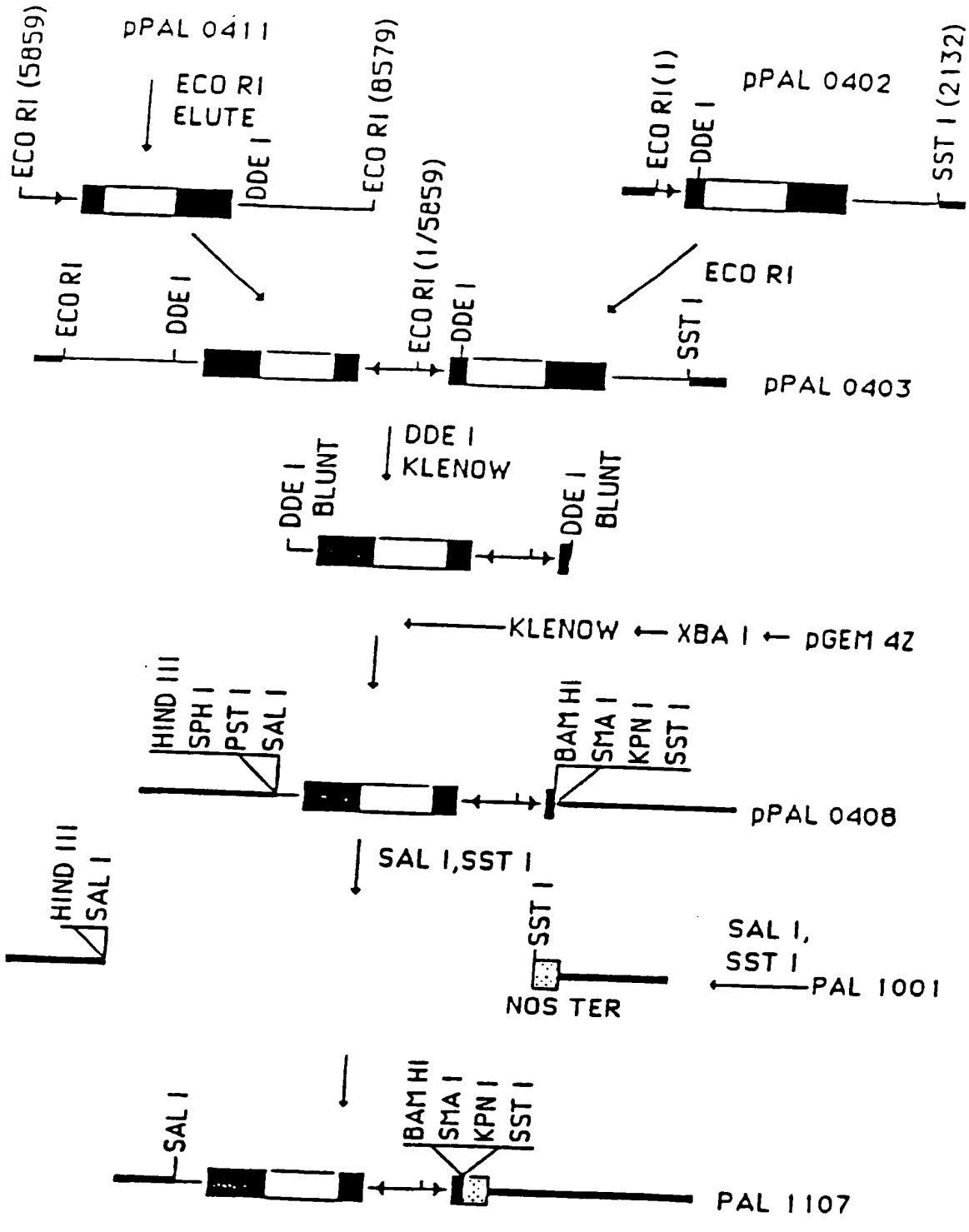
FIGURE 6

SEGREGATION OF BINARY CRYPTOCYTOTOXICITY GENES IF BOTH GENES ARE LOCATED ON THE SAME CHROMOSOME OF A CHROMOSOME PAIR IN THE ISOGENIC MALE STERILE LINE



SUBSTITUTE SHEET

FIGURE 7A



SUBSTITUTE SHEET

FIGURE 7B

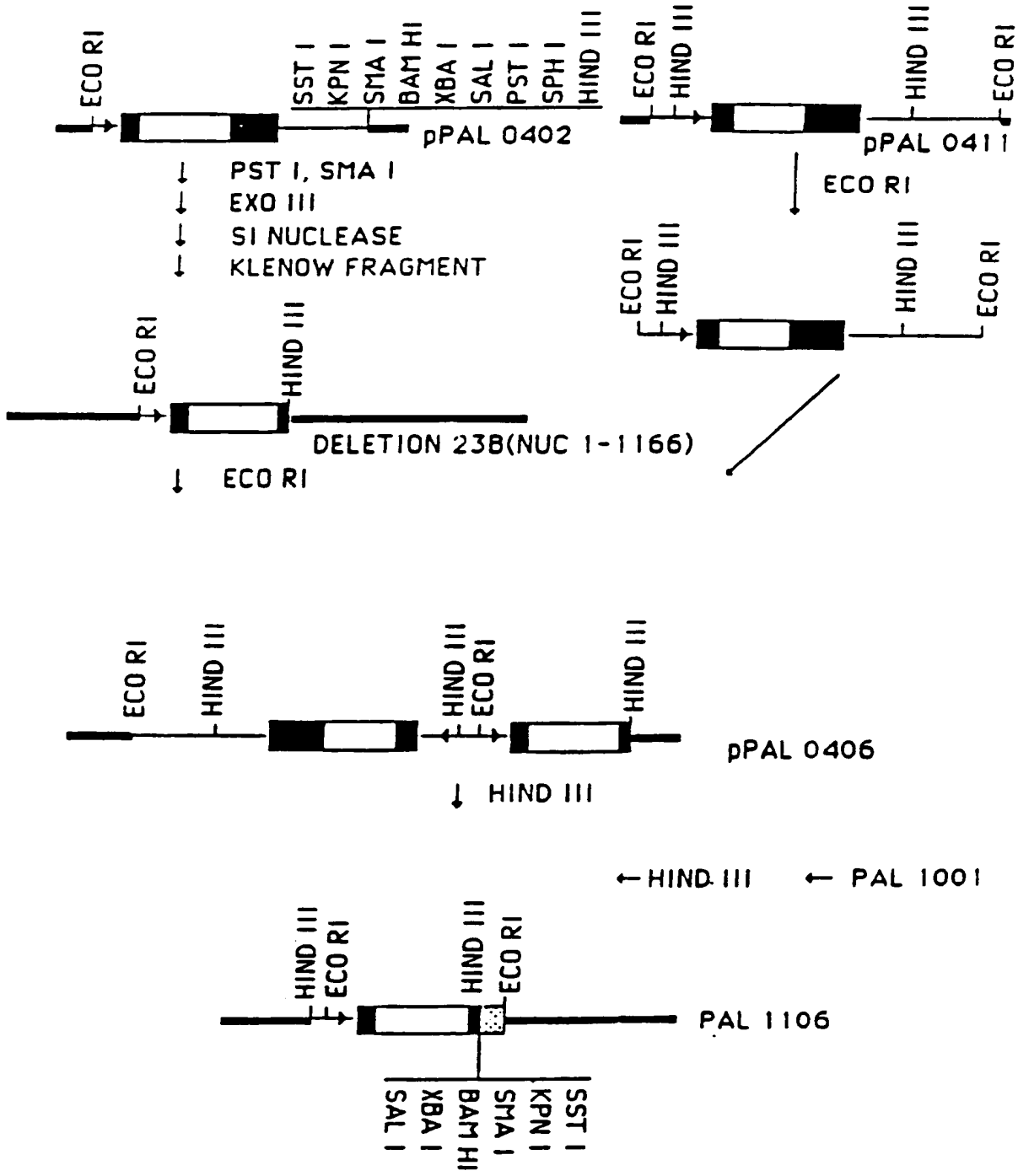

ECO RI
SST I
KPN I
SMA I
BAM HI
XBA I
SAL I
PST I
SPH I
HIND III
pPAL 0402
PST I, SMA I
EXO III
S1 NUCLEASE
KLENOW FRAGMENT
DELETION 23B(NUC 1-1166)
ECO RI
pPAL 0411
HIND III
pPAL 0406
HIND III
PAL 1001
PAL 1106

FIGURE 7C

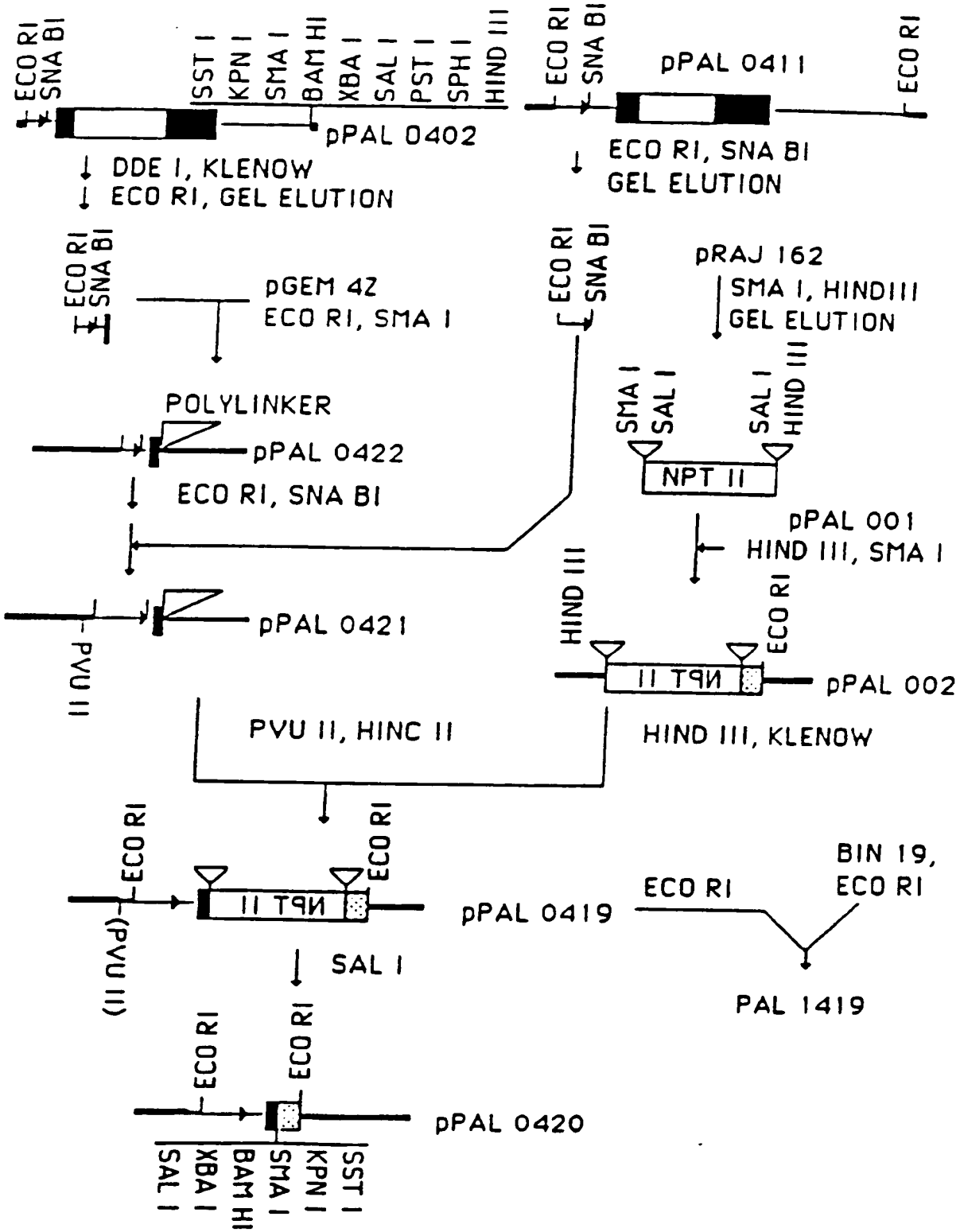

ECO RI
SNA BI
SST I
KPN I
SMA I
BAM HI
XBA I
SAL I
PST I
SPH I
HIND III
pPAL 0402
DDE I, KLENOW
ECO RI, GEL ELUTION
pPAL 0411
ECO RI, SNA BI
GEL ELUTION
pGEM 4Z
ECO RI, SMA I
pRAJ 162
SMA I, HINDIII
GEL ELUTION
POLYLINKER
pPAL 0422
ECO RI, SNA BI
NPT II
pPAL 001
HIND III, SMA I
pPAL 0421
PVU II
pPAL 002
HIND III, KLENOW
PVU II, HINC II
pPAL 0419
BIN 19,
PAL 1419
SAL I
pPAL 0420

FIGURE 7D

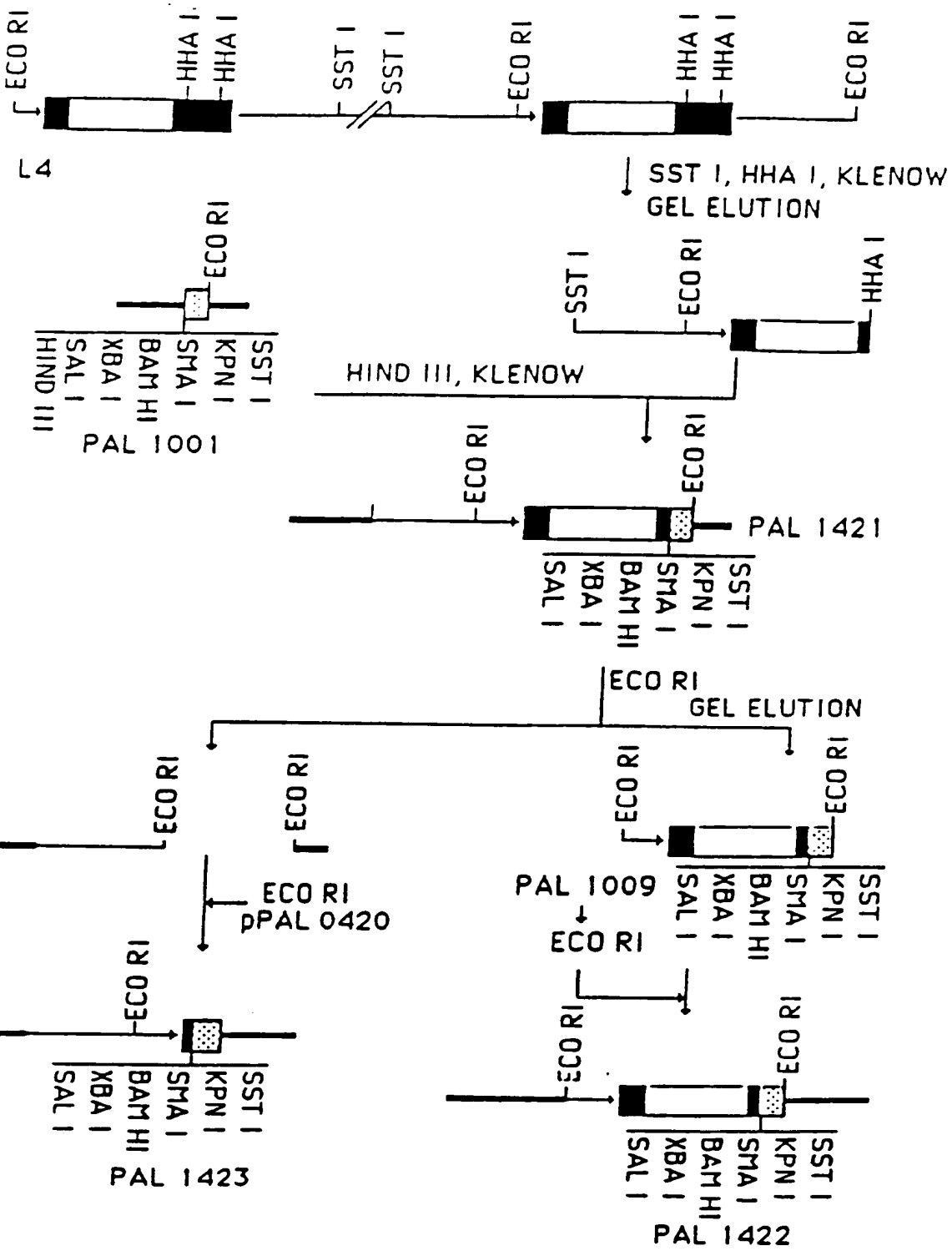

ECO RI
HHA I
HHA I
SST I
SST I
ECO RI
HHA I
HHA I
ECO RI
L4
SST I, HHA I, KLENOW
GEL ELUTION
ECO RI
HIND III
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
PAL 1001
SST I
ECO RI
HHA I
HIND III, KLENOW
ECO RI
ECO RI
PAL 1421
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
ECO RI
GEL ELUTION
ECO RI
ECO RI
ECO RI
pPAL 0420
ECO RI
ECO RI
PAL 1009
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
ECO RI
ECO RI
ECO RI
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
PAL 1423
ECO RI
ECO RI
SAL I
XBA I
BAM HI
SMA I
KPN I
SST I
PAL 1422

FIGURE 7E

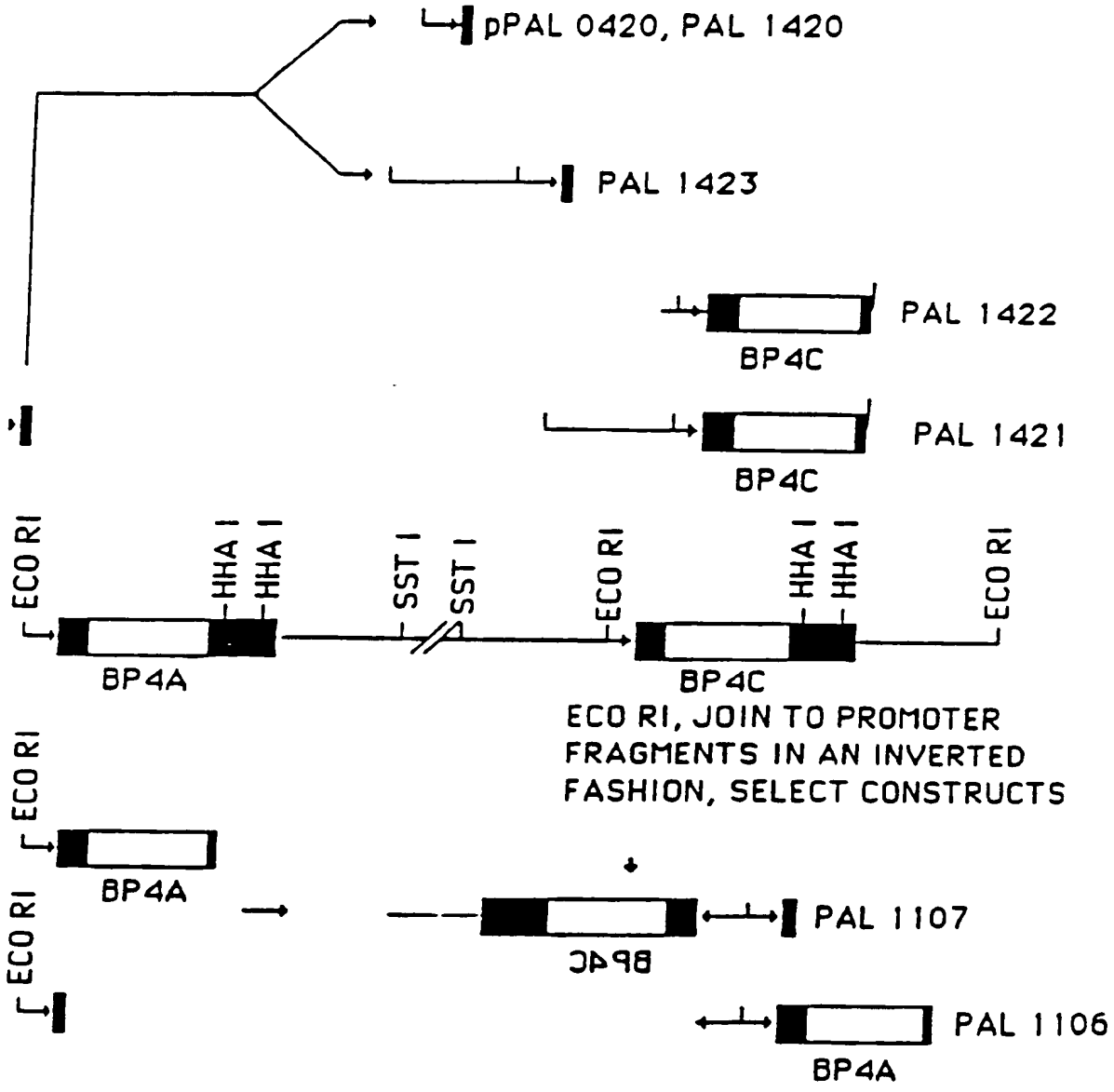

pPAL 0420, PAL 1420
PAL 1423
PAL 1422
BP4C
PAL 1421
BP4C
ECO RI
HHA I
HHA I
SST I
SST I
ECO RI
HHA I
HHA I
ECO RI
BP4A
BP4C
ECO RI, JOIN TO PROMOTER FRAGMENTS IN AN INVERTED FASHION, SELECT CONSTRUCTS
ECO RI
BP4A
ECO RI
PAL 1107
ECO RI
PAL 1106
BP4A

FIGURE 8

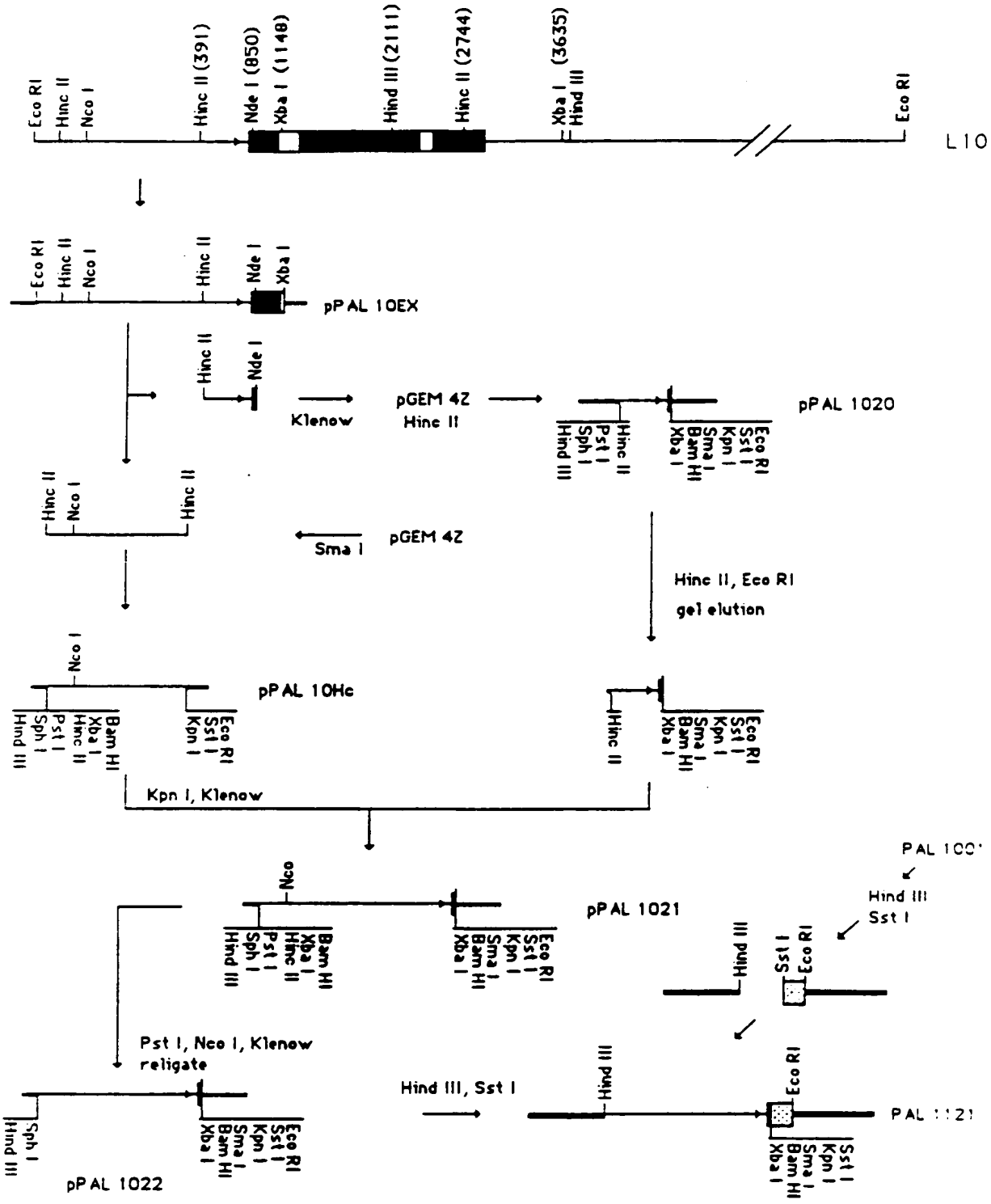

Eco RI
Hinc II
Nco I
Hinc II (391)
Nde I (850)
Xba I (1148)
Hind III (2111)
Hinc II (2744)
Xba I (3635)
Hind III
Eco RI
L10
pPAL 10EX
Klenow
pGEM 4Z
Hinc II
pPAL 1020
Sma I
pGEM 4Z
Hinc II, Eco RI
gel elution
pPAL 10Hc
Kpn I, Klenow
PAL 100
Hind III
Sst I
pPAL 1021
Pst I, Nco I, Klenow
religate
Hind III, Sst I
pPAL 1022

FIGURE 9

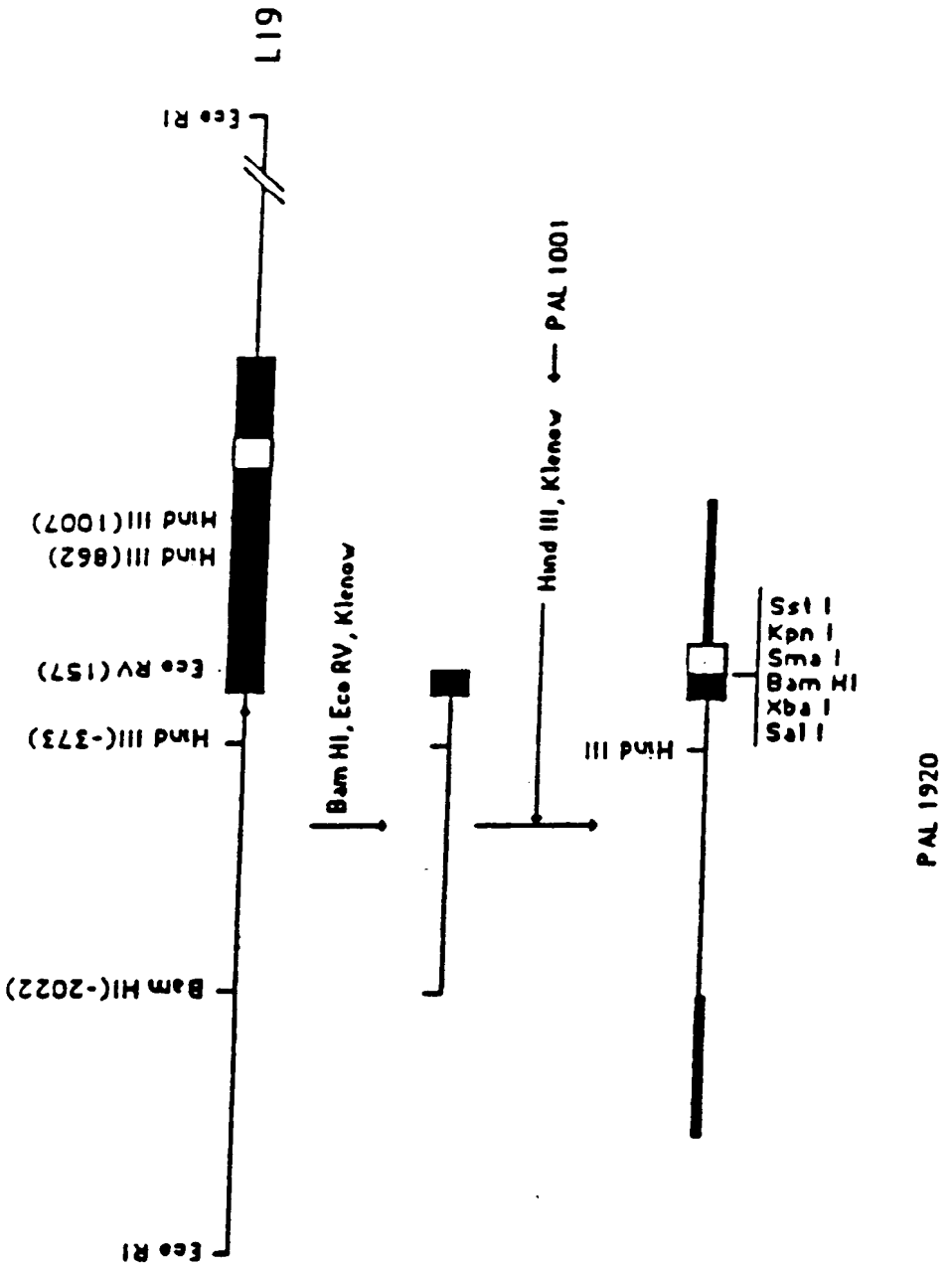
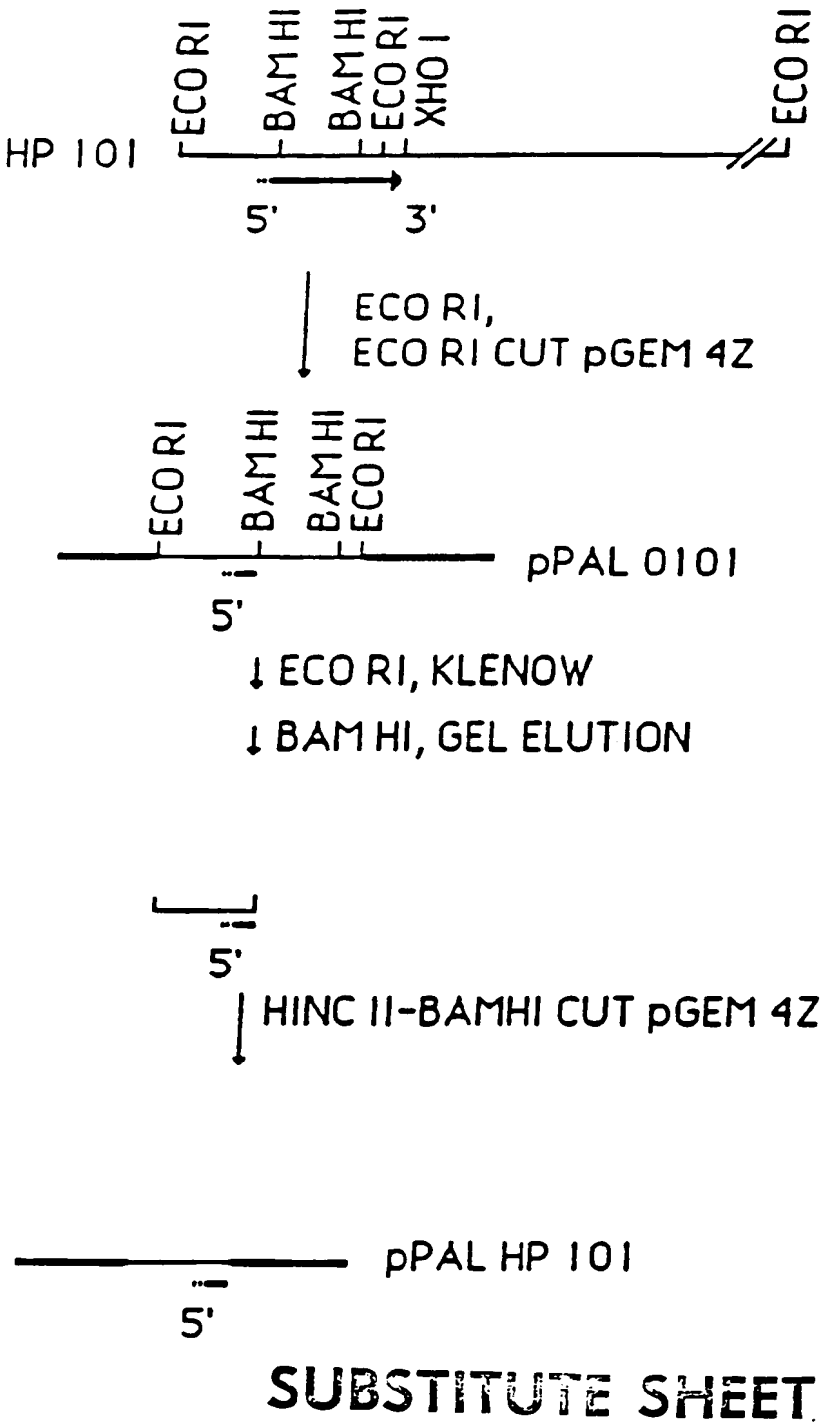

L19
Eco RI
Hind III (1007)
Hind III (862)
Eco RV (157)
Hind III (-373)
Bam HI (-2022)
Eco RI
Bam HI, Eco RV, Klenow
Hind III, Klenow
PAL 1001
Hind III
Sst I
Kpn I
Sma I
Bam HI
Xba I
Sal I
PAL 1920

FIGURE 10


ECO RI
BAM HI
BAM HI
ECO RI
XHO I
ECO RI
HP 101
5'
3'
ECO RI,
ECO RI CUT pGEM 4Z
ECO RI
BAM HI
BAM HI
ECO RI
pPAL 0101
5'
ECO RI, KLENOW
BAM HI, GEL ELUTION
5'
HINC II-BAMHI CUT pGEM 4Z
pPAL HP 101
5'

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 91/00255

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82 ; C12N15/55 ; C12N15/53 ; A01H1/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	PLANT SCIENCE vol. 46, no. 3, 1986, pages 195 - 206; BUDAR, F., ET. AL: 'Introduction and expression of the octopine T-DNA oncogenes in tobacco plants and their progeny ' see page 203	12
P,X	EP,A,412 911 (PLANT GENETIC SYSTEMS) February 13, 1991 see page 6, line 27 - line 28 see page 11, line 15 - line 32	13-16

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 OCTOBER 1991	05. 11. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	PHYSIOL. PLANT. vol. 79, no. 2, June 1990, PART 2 page A27; SITBON F., ET. AL: 'Transgenic tobacco plants overproducing IAA display abnormal growth and development ' see abstract 152	1-12
A	WO,A,8 910 396 (PLANT GENETIC SYSTEMS) November 2, 1989 see page 11, line 15; claims 5,12	1-24
A	EP,A,329 308 (PALADIN HYBRIDS) August 23, 1989 see the whole document	1-24

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

CA 9100255
SA 49356

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

15/10/91

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-412911	13-02-91	AU-A-	6068890	11-03-91
		WO-A-	9102069	21-02-91
		JP-T-	3503004	11-07-91
WO-A-8910396	02-11-89	AU-A-	3537189	24-11-89
		EP-A-	0344029	29-11-89
		JP-T-	2503988	22-11-90
EP-A-329308	23-08-89	AU-A-	2963289	03-08-89